Genetic Characterization of Invasion and Hybridization:

A Bittersweet (*Celastrus* spp.) Story

BY

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B.S., University of Illinois at Chicago, 2005

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biological Sciences
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

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This dissertation is dedicated to my aunt, Asmar Talya.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Mary Ashley, for giving me the opportunity to pursue a graduate degree and the guidance she has provided along the way. I would like to thank my collaborators, Dr. Noel Pavlovic and Dr. Stacey Leicht-Young, for their encouragement and friendship. I thank Dr. Henry Howe for his guidance dating back to my undergraduate education. I also acknowledge Dr. Boris Igic and Dr. Jeremie Fant for their support.

The work presented in this dissertation was funded by the US Geological Survey, National Park Service through the Great Lakes Research and Education Center, Chicago Wilderness, Illinois State Academy of Science, and University of Illinois at Chicago. I also acknowledge the financial support provided to me by the University of Illinois at Chicago.

Sample collection, data collection, data analysis, and communication of the dissertation were aided by Regina Morgan, Karen Zaya, Dr. Hormoz BassiriRad, Scott Price, Sarah Hicks, Thomas Rawinski, Ron Lance, Dr. Saji Abraham, Jim Scios, Noranne Magee, Jack Gibbons, John Podejko, James Rollins, Sam Zagone, Dr. Jennifer Ison, Dr. Paul Gulezian, John Wilk, Janet Backs, Emi Kuroiwa, Eun Sun Kim, Jason Palagi, Andy Raduski, James Daley, Chris Hetrea, Kirsten Dittel, Dominick Fosco, Iryna Shak, Patricia Majchrowski, Daniel Reyes, Elias Lawal, Claire Bridges, Dr. Kevin Feldheim, Dr. Stuart Wagenius, Dr. Joy Marburger, Bob Streitmatter, William Moorehead, John Balaban, and numerous scientists, managers, and stewards from the National Park Service, state and county park systems, and forest preserves.

Finally, I am grateful for the personal support provided by my extended family and friends. The list of important people is too long to fully enumerate here. However, I extend special thanks to Lodia Zaya, Yonan Zaya, Karen Zaya, Asmar Talya, William Zaya, Helen Talya, Odisho Youkhana, Ashur Youkhana, and Regina Morgan.

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TABLE OF CONTENTS

CHAPTER PAGE

1. GENETIC CHARACTERIZATION OF HYBRIDIZATION IN *CELASTRUS* FOLLOWING INVASION IN THE EASTERN USA 1
1.1 Introduction 1
1.2 Methods 3
1.2.1 Study species 3
1.2.2 Sample collection 4
1.2.3 Development of microsatellite loci 6
1.2.4 Microsatellite data collection 7
1.2.5 Hybrid identification 9
1.2.6 Restriction fragment length polymorphism (RFLP) 9
1.2.7 Hybrid reproductive potential 10
1.3 Results 11
1.4 Discussion 19
1.5 References 23

2. REDUCED REPRODUCTIVE SUCCESS IN A NATIVE VINE DUE TO REPRODUCTIVE INTERFERENCE FROM AN INTRODUCED CONGENER 29
2.1 Introduction 29
2.2 Methods 31
2.2.1 Study species 31
2.2.2 Study site and sampling 32
2.2.3 Male floral output 34
2.2.4 Manipulative hand-crosses 34
2.2.5 Comparing fertilization and hybridization rate between species 34
2.2.6 Predictors of hybridization rate in *C. scandens* 35
2.2.7 Data analysis 36
2.2.7.1 Equation 1 36
2.3 Results 37
2.3.1 Staminate floral output and hand-crosses 37
2.3.2 Fertilization rate in *C. scandens* and *C. orbiculatus* 38
2.3.3 Hybridization rate in *C. scandens* and *C. orbiculatus* 38
2.3.4 Predictors of hybridization rate in *C. scandens* 40
2.4 Discussion 40
2.5 References 48

TABLE OF CONTENTS (continued)

CHAPTER PAGE

3. MISLABELING OF AN INVASIVE VINE (*CELASTRUS ORBICULATUS*) AS A NATIVE PLANT (*C. SCANDENS*) IN HORTICULTURE 52
- 3.1 Introduction 52
- 3.2 Methods 54
  - 3.2.1 Study species 54
  - 3.2.2 Sampling 55
  - 3.2.3 Genetic analysis 57
  - 3.2.4 Statistical analysis 58
- 3.3 Results 58
- 3.4 Discussion 59
- 3.5 References 66

4. CARBON AND NITROGEN ENRICHMENTS CHANGE THE EFFECTS OF COMPETITION ON BIOMASS ALLOCATION IN A NATIVE (*CELASTRUS SCANDENS*) AND INVASIVE LIANA (*C. ORBICULATUS*) 70
- 4.1 Introduction 70
- 4.2 Methods 72
  - 4.2.1 Study species 72
  - 4.2.2 Experimental design 72
  - 4.2.3 Data analysis 74
- 4.3 Results 75
- 4.4 Discussion 78
- 4.5 References 82

APPENDIX 85

VITA 89

LIST OF TABLES

TABLE PAGE

I: Microsatellite primers 8

II: Summary statistics for microsatellite loci in two of the largest populations sampled for each species 13

III: Location and field identification of individuals later determined to be hybrids 14

IV: Microsatellite genotypes for named *Celastrus* varieties....61

V: MANOVA summary for biomass of different tissues in *C. scandens* 79

VI: MANOVA summary for biomass of different tissues in *C. orbiculatus*....79

VII: Sample sizes, locations, and species identities of *Celastrus* across the USA 85

LIST OF FIGURES

FIGURE PAGE

1.1: *Celastrus* collection sites 5

1.2: Inflorescences of a pistillate *Celastrus* hybrid 15

1.3: Fruit capsule colors 16

1.4: Scanning electron microscope image of pollen from a hybrid individual 17

1.5: Distribution of pollen grain widths for two staminate hybrids 18

2.1: Spatial distribution of focal *Celastrus* individuals at the Protage Lakefront study site 33

2.2: Mean staminate floral output per day. 39

2.3: Relationship between hybridization rate and distance to the nearest interspecific male in *C. scandens* 41

2.4: Hybridization rate in *C. scandens* versus the interaction of distance to the nearest interspecific male and $\log_{10}$ floral output of the nearest co-flowering interspecific male 42

3.1: Distribution of *Celastrus* vendors 56

3.2: Prices for *C. scandens* and *C. orbiculatus* purchased at Midwestern nurseries 62

4.1: Relationship between root-to-shoot ratio and the interaction of carbon dioxide concentration and competition treatment in *C. scandens* 76

4.2: Relationship between root-to-shoot ratio and the three-way interaction of carbon dioxide concentration, nitrogen level, and competition treatment in *C. orbiculatus* 77

LIST OF ABBREVIATIONS

bp	base pair
Co.	county
cpDNA	chloroplast DNA
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
GLM	generalized linear model
GLMM	generalized linear mixed model
GPS	global positioning system
log	logarithm
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RGR	relative growth rate
s.e.	standard error of the mean
SEM	scanning electron microscope
Taq	*Thermus aquaticus* DNA polymerase
USA	United State of America
USD	United States dollar

SUMMARY

Ecologists have widely recognized the effects of human-mediated species introductions. Rapidly spreading introduced species, or invasive species, have substantial economic costs, alter ecosystem processes such as succession and nutrient cycling, and harm human health. Also, invasive species often play a role in the loss of native biodiversity. The extent to which introduced species negatively affect native species has more recently come under question, and this issue remains controversial.

Increasingly researchers have begun to appreciate role of reproductive interference, such as hybridization, during species invasions. Hybridization associated with species introductions can accelerate declines of native species. Reproductive interactions during invasion are especially harmful to native populations because they display positive density dependence, whereas resource competition follows a negative density dependence pattern. Additionally, in sessile organisms such as plants the physical scale over which reproductive interactions occur can far exceed the scale of competitive. Finally, reproductive interactions can intensely target a single or small set of species, whereas the effects of resource competition are scattered across an ecological community.

One objective of my dissertation research was to determine if the decline of a North American vine (American bittersweet, *Celastrus scandens*) in the eastern portion of its range is related to reproductive interference (including hybridization) from an introduced congener (oriental bittersweet, *C. orbiculatus*). Chapters 1 and 2 address this question.

In the first study I used genetic markers and field observation to survey individuals across the USA to determine the prevalence of hybrids, and whether hybrid swarms are the driving force behind the invasion by *C. orbiculatus*. I found that hybrids are widespread, but only eight percent of non-native genotypes surveyed were hybrids. Although established hybrids were uncommon, other evidence in our study suggested that gene flow and

reproductive interference may be important factors in the decline of *C. scandens*. Unidirectional pollen flow was evident from genetic analysis of a maternally inherited marker, where all 20 hybrids tested in this study showed the cpDNA signature of *C. scandens*. When asymmetry in pollen flow favors male fecundity in an introduced species, declines in a native species are greatly accelerated.

In Chapter 2 I investigated the reproductive interaction between the two bittersweet species in more detail at a field site where they both occur and reproduce. I evaluated the effect of proximity and floral output of conspecific and interspecific males on fertilization and hybridization rates in the two species. I did not find evidence that *C. orbiculatus* prevents fertilization of *C. scandens* ovules, but genetic tests of seedlings provide strong evidence for reproductive interference. Fifty-one percent of *C. scandens* seeds were hybrids, compared to 1.6% of *C. orbiculatus* seeds. If hybrid seedlings are not considered as contributors to fitness, it is plausible that the decline in the native vine in the presence of the introduced species can be tied to the reproductive effort or opportunity wasted on hybrids. Hybridization rate in *C. scandens* was negatively associated with distance to the nearest interspecific individual, demonstrating that efforts to conserve that native must reduce exposure to *C. orbiculatus*. The degree of isolation required is greater than conventional cases where only competition is considered, as reproductive interference in this system can be substantial at distances as great as 100m.

The second major objective of dissertation work was to examine how anthropogenic factors contribute to invasion by *C. orbiculatus*, and expected effects in the future. Chapter 3 dealt with human commerce, while Chapter 4 dealt with anthropogenic alteration of biogeochemical cycles.

Many introductions have been the direct result of human cultivation, including approximately 85% of invasive woody plants in North America. *Celastrus orbiculatus* was

originally introduced to North America through horticulture, and continues to be used as an ornamental plant. There has been an increasing emphasis on using native plants, including *C. scandens*, as ornamentals because the industry recognizes the problem of invasive plants. However, mislabeling of products plant and animal products has been found in some cases, and there was reason to believe that it may be a problem with *Celastrus*. In Chapter 3, I used genetic markers to test the species identity of commercially available plants marketed as "American bittersweet" or "*Celastrus scandens*" and found that the majority of samples and named varieties obtained were actually *C. orbiculatus*. The substitution may occur intentionally because *C. orbiculatus* is easier to grow and has a long history in horticulture, or unintentionally because growers may confuse the species. Mislabeled *C. orbiculatus* was significantly less expensive than *C. scandens*, giving even well-meaning consumers the incentive to propagate and spread the introduced species. Human commerce is amongst the most important dispersal agents of introduced species, and understanding its role in the continuing spread of *C. orbiculatus* is essential in any large scale attempt to control the spread of the introduced vine and its negative effect on natural communities, and *C. scandens* in particular.

Two especially pervasive human-mediated changes to global biogeochemical cycles are the alterations of the nitrogen and carbon cycles. Carbon dioxide and reactive nitrogen are both important nutrients for terrestrial, aquatic, and marine ecosystems, and modification of their availability can affect several aspects of ecological communities, such as net primary productivity and community composition. In Chapter 4, I used experimental trials in a greenhouse setting to simulate the elevated atmospheric carbon dioxide and increased nitrogen deposition expected in the future. I tested hypotheses regarding differential responses of *C. orbiculatus* and *C. scandens* to the increased availability of carbon dioxide and nitrogen, and the interactive effects of elevated carbon dioxide and nitrogen. The total biomass of neither species responded to either treatment. Biomass allocation in *C. scandens* significantly responded to

carbon dioxide and competition treatments, while biomass allocation in *C. orbiculatus* responded to nitrogen, carbon dioxide, and competition treatments. The results suggest increased phenotypic plasticity in *C. orbiculatus*, a factor that is often associated with successful invaders, and that *C. orbiculatus* may be better able to cope with future changes to biogeochemical cycles.

Overall, the work presented in this dissertation reveals factors involved in the decline of *C. scandens* and successful invasion of *C. orbiculatus*. Reproductive interference, human commerce, and changing biogeochemical cycles likely played a role in the decline of the native vine and spread of the invasive congener in the past, and their influence will likely increase in the future.

1. GENETIC CHARACTERIZATION OF HYBRIDIZATION *CELASTRUS* FOLLOWING INVASION IN THE EASTERN USA

1.1 Introduction

Researchers have widely recognized the effects of human-mediated species introductions. Rapidly spreading introduced species ("invasive species") have substantial economic costs (Aukema et al. 2011), alter ecosystem processes such as succession (Rudgers et al. 2007) and nutrient cycling (Ehrenfeld 2003), and harm human health (Fumanal et al. 2007). The role of introduced species in the loss of native biodiversity is less well established. The widely accepted notion that introduced species have far-reaching negative affects on native species (Wilcove et al. 1998) has more recently come under fire, although the issue remains controversial (Gurevitch and Padilla 2004, Ricciardi 2004, Schlaepfer et al. 2011, Vitule et al. 2012).

Complex dynamics may arise when introduced species invade the range of congeners. The decline of native species in such cases may be due not only to straightforward processes such as competition, but also because of gene flow and hybridization (Rhymer and Simberloff 1996). Hybridization between native and introduced species is a serious concern for conservation because it can lead to the rapid decline of some native species, as evidenced by empirical studies (e.g. Anttila et al. 1998) and modeling (Huxel 1999, Wolf et al. 2001, Hall et al. 2006). The loss of a native species may be due to hybrid swarms, where hybrid individuals are more fit or numerous than the native parental species (Vila and D'Antonio 1998), or where pure lines of the native species are threatened by introgression (Bleeker et al. 2007, Boyer et al. 2008, Maschinski et al. 2010). Hybridization may also lead to the evolution of invasive taxa or genotypes that outcompete the native parental species (Ellstrand and Schierenbeck 2000). A native species or subspecies may decline even if hybrids are sterile, rare, or absent because of wasted reproductive effort from fertilization by heterospecific pollen (Mercure and Bruneau

2008), which is exacerbated in cases of asymmetric pollen flow (Buggs and Pannell 2006, Prentis et al. 2007) or when the introduced species greatly outnumbers the native (Burgess et al. 2008). As the negative consequences of hybridization are most pronounced for rare species and small populations (Levin et al. 1996), there exists the potential for a feedback where species introduction and hybridization--perhaps in conjunction with habitat loss or disruption--lead to decreased abundance of a native species, which may in turn increase the rate and impact of hybridization (Rhymer and Simberloff 1996). Alternatively, native taxa may decline with the introduction of a congener in the complete absence of hybridization (Saltonstall 2003), due to competitive exclusion which may be intense when interactions are with species that has a similar niche. Also, native taxa may be largely unaffected by hybridization if the rate is very low or if pollen flow is unidirectional from native staminate flowers to introduced pistillate flowers (Conesa et al. 2010).

The two *Celastrus* species in eastern North America provide an opportunity to study the role of a species introduction and hybridization in the decline of a native congener. *Celastrus scandens* L. (Celastraceae), commonly known as American bittersweet or American staff vine, is a liana native to the USA and southern Canada, from the Atlantic Ocean to the Rocky Mountains. *Celastrus orbiculatus* Thunb., commonly known as oriental or Asiatic bittersweet, is a liana native to eastern Asia that was introduced to the east coast of the USA in the nineteenth century, and has now spread over much of the range of *C. scandens*. Declines in *C. scandens* have been observed in parts of its range, especially where the invasion of *C. orbiculatus* is oldest (Steward et al. 2003, Leicht 2005). Hand-crossed hybrids have been created in controlled settings, and hybrid seedlings compare favorably to *C. scandens* seedlings with regard to dormancy time, growth rate, and maximum size (White and Bowden 1947, Pooler et al. 2002). Dreyer et al. (1987) speculated that wild hybrids may exist, and individuals with intermediate floral morphology are occasionally found (R. W. Lance *pers. comm.*; T. J. Rawinski *pers. comm.*).

The objective of this study was to determine if the decline of *C. scandens* is related to reproductive interference from *C. orbiculatus*. I used genetic markers and observation to survey individuals across the country to determine the prevalence of hybrids. My first goal was to test whether hybrid swarms are driving the invasion. If so, a large proportion of individuals surveyed will be hybrids, and hybrids will not show signs of reduced fecundity or vigor. Second, I assessed asymmetry in the direction of pollen flow by testing hybrid individuals at a maternally inherited (cpDNA) marker. If pollen flow was asymmetric, there would be a significant imbalance in the number of hybrids carrying the genetic signature indicative of one or the other parental species. The findings of this study will provide information on the causes and nature of decline of *C. scandens* in North America, as well as providing insights into factors that have promoted success of *C. orbiculatus*. The findings will be informative for any effort to conserve *C. scandens* or manage the spread of *C. orbiculatus* where the two species co-occur.

1.2 Methods

1.2.1 Study species

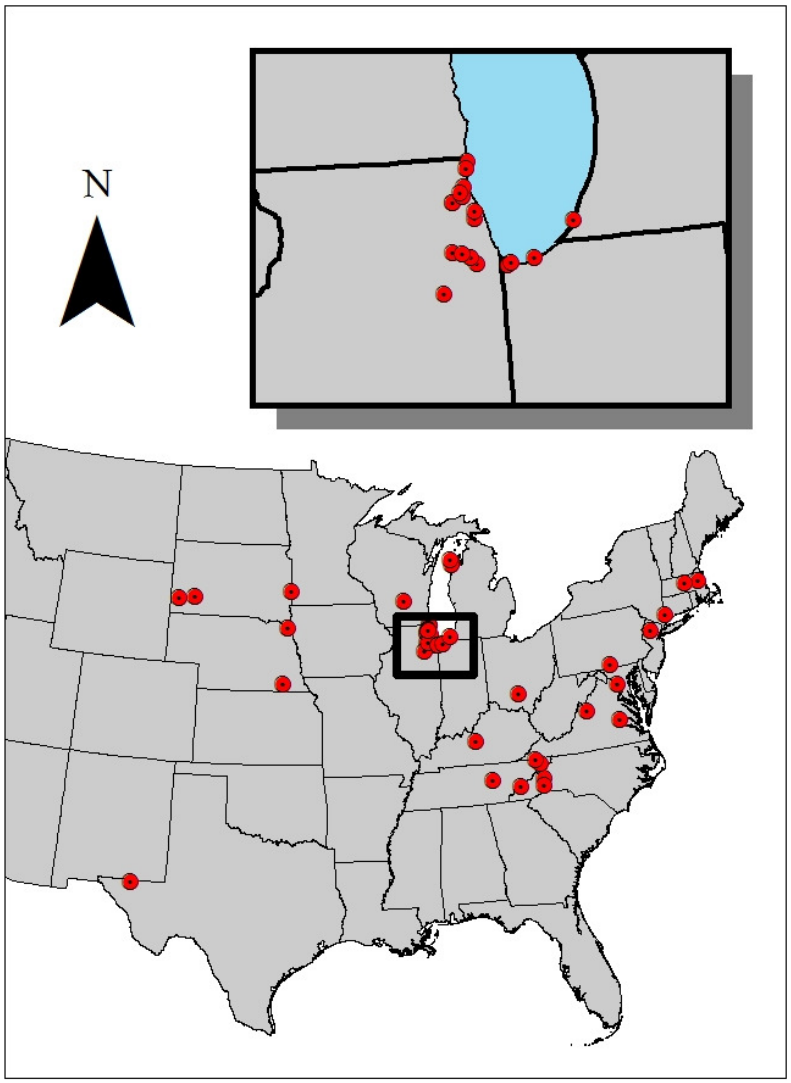
Celastrus is a genus of approximately 30 species found in Asia, Oceania, the Americas, and Madagascar (Hou 1955). The only species native to North America is *C. scandens*. *Celastrus scandens* is a liana usually found in open habitats. Its range extends from southern Quebec to South Dakota, south to western Texas through Georgia. The native range of *C. orbiculatus* is far eastern Asia, in Korea, Japan, and China. It is also a liana found in thickets and lowland slopes (Hou 1955), but can thrive in shaded habitat that would likely exclude *C. scandens* (Pavlovic and Leicht-Young 2011). Both species are usually dioecious, although rare individuals and populations displaying other breeding systems are known. Insects, particularly hymenoptera, pollinate both species, and birds are largely credited as the dispersers of the seeds that sit inside bright red fleshy fruits surrounded by an orange (*C. scandens*) or yellow (*C. orbiculatus*) capsule (Brizicky 1964).

Celastrus orbiculatus was introduced as an ornamental vine to the eastern USA in the 1860's. The first reports of it escaping into the wild came in the early twentieth century. By the mid-twentieth century it was widely recognized as a pest species spreading in the eastern USA (Patterson 1974). Land managers and foresters consider *C. orbiculatus* a troublesome species because it is a strong competitor that crowds out native vegetation, negatively affects productivity in forestry operations, and can alter natural successional trajectories (Fike and Niering 1999, Leicht-Young et al. 2007b). Notably, *C. scandens* has declined most in areas where invasion by *C. orbiculatus* is oldest and most extreme (Dreyer et al. 1987, Stewart et al. 2003, Leicht 2005, R. I. Bertin *pers. comm.*), although it is not clear whether or not there is a direct relationship between the two patterns. *Celastrus scandens* is listed as a species of special concern in several states in the eastern portion of its historical range. In Delaware it may be extirpated, while it is listed as a vulnerable species in New York, a threatened species in Massachusetts, and an endangered species in North Carolina.

1.2.2 Sample collection

I sampled *Celastrus* at a total of 43 sites in the eastern USA (Fig. 1.1; Table 5, Appendix). Two sites (Blue Ridge Parkway, NC and the Worcester County, MA site) were suspected *a priori* to have hybrids, based on intermediate floral morphology. I personally sampled 23 sites, collecting between two and 102 samples per site. When both species could be identified by differences in reproductive structures, I sampled both species. For larger populations when a census was not possible, collection efforts were spread out spatially to avoid drawing multiple samples from genets that may have multiple ramets. For an additional 20 sites collection was conducted by other scientists and naturalists, the majority of whom were National Park Service scientists making collections on National Park Service (NPS) land. I requested leaf samples from 10 individuals of each *Celastrus* species available at the NPS site. In all cases, leaf samples were immediately desiccated using silica gel or calcium sulfate (W.A. Hammond Drierite Co. Ltd., Xenia, OH, USA).

Figure 1.1: *Celastrus* collection sites. The inset shows an area of especially thorough sampling in northeastern Illinois, northwestern Indiana, and southwestern Michigan. In total, 43 sites were included in this study.



In addition to population sampling, I used leaf samples from hand-crossed hybrids as a control, to test the ability of my genetic analyses to identify hybrids. Hand-crosses were performed in the spring of 2006 at the Indiana Dunes National Lakeshore. Pistillate flowers were bagged with bridal mesh before opening. After hand-pollination with interspecific pollen, flowers remained bagged until fruit development. The resulting fruits were collected in October and germinated in a greenhouse setting after cold stratification, using the procedure described by Young and Young (1992).

1.2.3 Development of microsatellite markers

In order to isolate nuclear microsatellite markers for *Celastrus* I used an enrichment method developed by Glenn and Schable (2005). As a template, I used DNA extracted from one individual of each species. The species identity was determined by floral morphology. DNA was extracted by using the DNeasy Plant Mini Kit (Qiagen), and 20 µL of the final extraction product was used to digest DNA into smaller strands, using the restriction enzymes *Rsa*I and *Xmn*I. I ligated a double-stranded linker (SuperSNX) to the DNA fragments. The linker provides the binding site for primers in later PCRs and allows for cloning of the fragments. I used Dynabeads (Invitrogen), in conjuction with biotinylated di-, tri-, and tetranucleotide microsatellite probes, to enrich the microsatellite-containing DNA. Dynabeads are steptavidin-coated magnetic beads, and it is the steptavidin that binds to the biotinylated probes. A magnetic particle-collecting unit was used to capture beads while DNA fragments that were not bound to the microsatellite probes were washed away. The probes were removed from the DNA fragments by denaturing. To increase the amount of enriched DNA, fragments were amplified using PCR. The enriched DNA fragments were cloned using the TOPO-TA Cloning Kit (Invitrogen). I directly amplified DNA from colonies that showed signs of successful incorporation of *Celastrus* DNA fragments by PCR using M13 primers. The PCR products were cleaned using NucleoFast 96 PCR Plates (Macherey-Nagel), following the manufacturer's protocol. The BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) was

used to sequence the cleaned PCR fragments on an ABI 3730 DNA Analyser (Applied Biosystems). I assembled and visually inspected sequences for microsatellites using Sequencher 4.6 (GeneCodes) software. The programs OligoCalc (Kibbe 2007) and Primer 3 (Untergasser et al. 2007) were used to develop primers from the sequences flanking microsatellites. Further details of the protocol used to isolate the microsatellite markers can be found in Pauls et al. (2007).

1.2.4 Microsatellite data collection

I designed PCR primers for five loci (Table I). Three of the sequences used to develop the primers originated from *C. scandens*, and the other two were from *C. orbiculatus*. For fluorescent labeling, forward primers were synthesized with an M13 tail (Schuelke 2000). Genomic DNA was extracted from 20-25 mg of ground leaf material using the DNeasy Plant Mini Kit (Qiagen). PCR was conducted using 10-50 ng of genomic DNA in 10 µL volume containing 0.5 mM dNTP mix (Denville Scientific), 0.05 – 0.1 µM of the forward primer with the fluorescently labeled M13 (-21) universal primer extending from the 5' end, 0.33–0.6 µM of reverse primer, 0.16 µM fluorescently labeled M13 primer, 2.5 µg/ µL bovine serum albumin, and 0.025 U/ µL of Taq polymerase (Biotherm Taq; eEnzyme) with Biotherm buffer (originally 10x, diluted to 1x). I conducted thermal cycling under the following conditions: 94ºC for 5 minutes, 35 cycles of denaturing (94ºC), annealing (see Table I for temperatures), and elongation (72 ºC) with each step lasting 30s, and a final extension at 72 ºC for 5 minutes. Fragment sizes of PCR product (using 1.0 – 1.5 µL) were analyzed with the ABI 3730 DNA Analyser, using a LIZ500 ladder (Applied Biosystems). All microsatellite genotypes were scored by analyzing the raw data using Applied Biosystem GeneMapper software, version 3.7. 0

Ramets representing mutiple samples of the same genet were identified using the *allelematch* package in R version 2.15.1 (Galpern et al. 2012, R Core Team 2012), allowing one mismatched locus. I checked the microsatellite data for evidence of null alleles using

Table I: Microsatellite primers. Primer sequences are in 5'-3' order. The fluorescently labeled M13 (-21) universal primer was added to the 5' end of each forward primer.

Locus	Source species	Forward primer sequence	Reverse primer sequence	Annealing temperature (ºC)	Repeat motif	Size range (bp)
CEOR7003	*C. orbiculatus*	ACCCGGTTTCGTCTCTCTTT	GTGGCCGTCTTCGTTATCTC	52	$(TC)_{13}$ … $(TTC)_{12}$	204-248
CEOR7004	*C. orbiculatus*	AAAACGGATCCATCGAAACA	CCATTTTGCGCACTCTCTCT	54.5	$(GA)_{16}$	145-177
CESC002	*C. scandens*	TGGTTGCAGGAATTTGAAGA	TGAAAGCAGGAAAAGTGGACA	49	$(TC)_{18}$	215-245
CESC003	*C. scandens*	CTGCAGATCAACCAATGATG	GTTGCCGTTTTATGGGCTTA	53.5	$(TG)_{10}$ … $(AG)_{10}$	184-224
CESC006	*C. scandens*	GGCTATCCCAGTTTGGTTGA	GAAGCCACTTTGTGACAGCA	53.5	$(ATAC)_6$	201-236

Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). I used Arlequin version 3.5.1.2 to test for deviations from Hardy-Weinberg equilibrium (Excoffier and Lischer 2010).

1.2.5 Hybrid identification

Species assignments and hybridization were evaluated using the programs STRUCTURE version 2.3 (Falush et al. 2007) and NewHybrids version 1.1 beta (Anderson and Thompson 2002). No *a priori i*nformation on species identity was included in the analyses. Both programs implement a Bayesian clustering approach and Markov chain Monte Carlo simulations to provide estimates of an individual's identity using posterior probabilities. The assumptions for each program and the interpretation of probabilities are somewhat different. NewHybrids gives the probability that each individual belongs to one of the two parental species or to the hybrid category or categories (several hybrid categories can be defined). The posterior probabilities of STRUCTURE give the estimated proportion of an individual's genome that originated from each cluster (the user defines the number of clusters, K). The two models are similar when specifying two clusters for the STRUCTURE model. For STRUCTURE analysis, I used the admixture model assuming correlated allele frequencies and set $K = 2$, corresponding to the two species. I averaged data from three runs, each with 250,000 iterations after an initial burn-in of 50,000 iterations. I used a threshold of $q < 0.85$, the posterior probability of an individual genotype belonging to a single genetic cluster, to categorize individuals as hybrids. For NewHybrids, data were averaged across three separate runs, each with 350,000 iterations after an initial burn-in of 50,000 iterations. Each individual was assigned to the genotype class with the maximum likelihood. Three classes were established in the model (two parental classes, and hybrids) and no individual had a maximum probability less than 0.6. Jeffreys' priors were used for the allele frequency (θ) and mixing proportion (π) parameters.

1.2.6 Restriction fragment length polymorphism (RFLP)

I used a chloroplast DNA (cpDNA) RFLP marker to identify the maternal species of hybrid individuals. The RFLP marker utilizes a difference in the *rbcL* gene sequence (accessed

through GenBank, AY788194 and AY788195); *C. scandens* has one restriction site for the enzyme PvuII, while *C. orbiculatus* does not have any.

For the RFLP test, a 1171 bp region of the chloroplast genome was amplified using the following primers: forward— 5' CTGGCGTTAAAGATTATAAATTGAC, reverse—5' CCTCCACCGAATTGTAGTACG. PCR was conducted using 10-50 ng of genomic DNA in 10 µL of PCR mix with the following reagents (final concentrations given): 0.5 mM dNTP mix (Denville Scientific), 0.5 µM each of the forward and reverse primers, 1.5 µg/ µL bovine serum albumin, and 0.025 U/ µL of Taq polymerase (Biotherm Taq; eEnzyme) with Biotherm buffer (originally 10x, diluted to 1x). The thermal cycling conditions were: 94ºC for 2 minutes, 35 cycles of denaturing (94ºC for 45s), annealing (59 ºC for 45s), and elongation (72 ºC for 90s), and a final extension at 72 ºC for 2 minutes. For restriction digest I used a 7.5 µL reaction containing 5 µL of PCR product , 0.625 U PvuII restriction enzyme (Fisher Scientific), and 0.75 µL Digestion buffer 2 (Fisher Scientific). The mixture was incubated at 37ºC for 1 hour. Fragment sizes were visualized under UV light on a 1.5% agarose gel containing ethidium bromide (3 µL EtBr / 100 mL gel). In *C. scandens*, two fragments were expected that were 1012 bp and 159 bp long, while an undigested 1171 bp long fragment was expected from *C. orbiculatus*. I included positive and negative controls (as well as 100 bp size standard) in each electrophoresis run.

1.2.7 Hybrid reproductive potential

Most hybrid individuals I encountered did not have reproductive structures, and many of the hybrids were sampled by other scientists on National Park Service lands. Three hybrid plants were identified in the field by reproductive structures and studied further. One of the individuals was a pistillate plant located in Cook Co., IL. The other two hybrid individuals were found and examined by T. J. Rawinski in Worcester Co., MA. In 2010 and 2011, I collected fruits from the pistillate hybrid and counted the proportion with fully developed seeds, the average number of viable seeds per fruit, and estimated the mean seed mass for each

individual by dividing the total mass of seeds collected by the number of seeds. I compared the proportion of fruits with fully developed seeds, mean number of seeds per fruit, and mean seed mass (at the plant level) for the hybrid individual to fruits collected from individuals at the Indiana Dunes National Lakeshore. Those fruits were collected in 2010 from individuals that were genetically identified as belonging to one of the parental groups (n = 21 and 14 for *C. orbiculatus* and *C. scandens*, respectively). The staminate flowers were collected in Worcester Co., MA during the spring of 2010, stored in 70% ethanol solution at 4°C, and examined at the University of Illinois at Chicago.

For preliminary light microscopy, pollen was stained with a mix of glycerol jelly and phenol blue and examined at 10x-100x. In preparation for the scanning electron microscope, anthers were sliced longitudinally and gradually dehydrated at room temperature in increasing concentrations of ethanol, in the following sequence: 75% ethanol for one hour, twice; 95% ethanol for 1 hour; 100% ethanol for 1 hour, three times. Following dehydration, samples went through critical point drying with carbon dioxide to avoid damage to the pollen grains' surface details. Samples were then mounted on aluminum stubs with double-sided tape and sputter coated with approximately 280 ångströms of gold. Samples were examined using a JEOL 5600LV SEM. Images at 450-2500x magnification were used to estimate pollen grain width. I compared the pollen from the hybrid individuals to the images posted by the Missouri Pollen Project (Bogler 2011), and the description in Dreyer et al. (1987) for the parental lineages. I noted the proportion of small and poorly developed pollen grains.

1.3 Results

Microsatellite data revealed that I sampled ramets from the same genet in multiple populations for both species. Clonal growth was at times impressive; one *C. scandens* genet was spread out over nearly 100 meters. I removed clones from further analyses, which substantially reduced sample sizes in a number of populations (Table 5, Appendix). Out of the

initial 654 samples (excluding control individuals), 475 unique genotypes remained in the analysis. There was no evidence of deviation from Hardy-Weinberg equilibrium or null alleles after removing clones and applying Bonferroni or Šidák corrections. Table II contains summary statistics for each locus at two of the largest sites where both species occur.

When testing for hybrid genotypes, the results from STRUCTURE and NewHybrids corresponded nearly perfectly. Both programs identified all 16 hand-crossed hybrids correctly. STRUCTURE identified 20 additional unique genotypes as hybrids, and NewHybrids identified 19 of these 20 individuals as hybrids. STRUCTURE classified one individual as a hybrid, while NewHybrids classified it as *C. orbiculatus*. Preliminary morphological assessment suggested the individual was *C. scandens*. I conclude that 20 of the 475 unique genotypes tested represent hybrids (4.2%). Post-hoc investigation of allele frequencies indicates that the two species had nearly unique sets of alleles at two loci (CESC006 and CEOR7004). The hybrid samples were collected from seven sites in four states (Table III). The two parental species co-occurred at four sites.

After RFLP analysis, all 20 hybrid individuals showed the restriction fragment pattern characteristic of *C. scandens* cpDNA, indicating that every hybrid individual arose from a *C. scandens* maternal lineage and *C. orbiculatus* was the pollen donor. Assuming the siring of each hybrid was an independent event, the 95% binomial confidence interval estimates that 81%-100% of hybrids arise from *C. scandens* pistillate plants (Agresti and Coull 1998).

I observed one pistillate hybrid individual, at the Bunker Hill site in Cook County, IL (Fig. 1.2). The individual had both axillary and terminal inflorescences, typical of *C. orbiculatus* and *C. scandens*, respectively. The ovary walls that develop into the capsules surrounding the aril were light orange or ochre, intermediate in color between the yellow typical of *C. orbiculatus* and the deep orange typical of *C. scandens* (Fig. 1.3). The individual flowered and set fruit profusely, demonstrating that hybrids can reach reproductive maturity and set fruit.

Table II: Summary statistics for microsatellite loci in two of the largest populations sampled for each species. N – sample size, A – number of alleles, H_o – observed heterozygosity, H_e – expected heterozygosity, P – p-value for deviation from HWE.

		Indiana Dunes NL					Warren Dunes SP				
Locus		N	A	H_o	H_e	P	N	A	H_o	H_e	P
CESC002	*C. orbiculatus*	44	11	0.727	0.766	0.019	21	10	0.905	0.842	0.304
	C. scandens	43	11	0.767	0.768	0.641	18	9	0.833	0.894	0.226
CESC003	*C. orbiculatus*	34	5	0.647	0.595	0.946	19	3	0.211	0.240	0.113
	C. scandens	49	11	0.796	0.838	0.112	17	10	0.882	0.852	0.748
CESC006	*C. orbiculatus*	45	3	0.556	0.527	0.181	21	3	0.810	0.633	0.368
	C. scandens	49	3	0.449	0.505	0.532	18	2	0.278	0.386	0.258
CEOR7003	*C. orbiculatus*	46	14	0.826	0.853	0.485	20	6	0.700	0.664	0.457
	C. scandens	51	7	0.863	0.820	0.492	17	7	0.764	0.772	0.470
CEOR7004	*C. orbiculatus*	43	9	0.651	0.596	0.010	22	7	0.909	0.748	0.483
	C. scandens	53	5	0.208	0.226	0.096	18	3	0.222	0.208	1.000

Table III: Location and field identification of individuals genetically determined to be hybrids. Individuals not listed here for which the species identity was initially unknown clustered with *C. scandens* or *C. orbiculatus*. Asterisks denote sites where the two progenitor species co-occur.

Location	State	Field identification	N
Bunker Hill	IL	Unknown	2
Bur Oak Woods	IL	C. orbiculatus	1
Ryerson Conservation Area *	IL	Unknown	3
Waterfall Glen	IL	*C. orbiculatus*	2
Worcester County, Utility right-of-way *	MA	Intermediate	5
Warren Dunes SP *	MI	*C. scandens*	1
Blue Ridge Parkway *	NC	Unknown	6

However, the fruits of the hybrid individual were much smaller than that of either parental lineage (fleshy arils 2-4 mm across the equatorial axis, compared to 6-8 mm for the parental species). Upon further inspection, I found that only 1.6% and 0% of fruits contained developed seeds in 2010 and 2011, respectively. In most fruits, seeds were not present, or were empty coats a small fraction of (less than one quarter) the size of a normal seed. Fruits from both parental species almost always contain at least one fully developed seed (in 2010, means of 99.8% and 99.4% for *C. orbiculatus* and *C. scandens*, respectively). Also, each fruit in the hybrid individual contained a single seed, while the seed to fruit ratio was much larger in the parental species (3.7 ± 0.2 seeds/fruit [1 s.e., n = 21] for *C. orbiculatus*, 3.1 ± 0.2 seeds/fruit [1 s.e., n = 14] for *C. scandens*). Mean mass of seeds from the hybrid was intermediate between the parental species (11.0 mg for the hybrid, 9.6 ± 0.4 mg [1 s.e., n = 21] for *C. orbiculatus*, 15.4 ± 0.8 [1 s.e., n = 14] mg for *C. scandens*). In total, I collected six seeds from the hybrid individual; one successfully germinated.

Figure 1.2: Inflorescences of a pistillate hybrid *Celastrus* individual. Hybrid pistillate and staminate individuals have both terminal panicles typical of *C. scandens* and axillary cymes typical of *C. orbiculatus*.

Figure 1.3: Fruit capsule colors. The ovary wall develops into a brightly colored capsule that surrounds the developing fruit until it dehisces to reveal the red aril. On the right and left are the deep orange and bright yellow capsules typical of *C. scandens* and *C. orbiculatus*, respectively. In the middle, capsules from a hybrid pistillate plant are intermediate in color and substantially smaller.

Scanning electron microscopy showed that size distribution of pollen grains collected from two staminate hybrids in Worcester County, MA was unimodal (Fig. 1.4 and 1.5), with a maximum at 16.2 µm. Distribution of pollen grain widths was skewed to the right (median = 16.7 µm, mean = 17.1 ± 0.06 µm [1 s.e., n = 946]). A large majority (92.1%) of pollen grains were less than 20 µm wide—the average pollen size for the two parental species—, and 78.4% of pollen was smaller than 18 µm—the lower limit of the typical range for the two parental species. Our observations, in combination with the observations of Dreyer et al. (1987) showing a positive correlation between pollen size and viability, suggest that most pollen from staminate hybrids is inviable. The shape of both the large and small pollen grains was similar to the shape typical of the parental species, tricolporate monads that are approximately spheroidal.

Figure 1.4: Scanning electron microscope image of pollen from a hybrid individual (1750x). The distribution of pollen grain widths was bimodal. The pollen grain in the top right of this image is typical of the larger group of pollen grains (approximately 23 µm wide), but the vast majority were substantially smaller (approximately 16 µm wide).

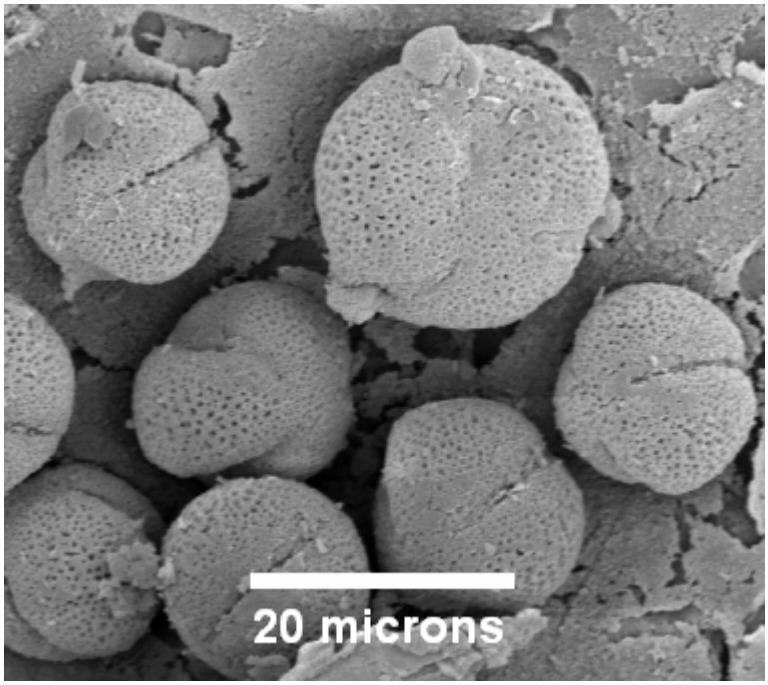
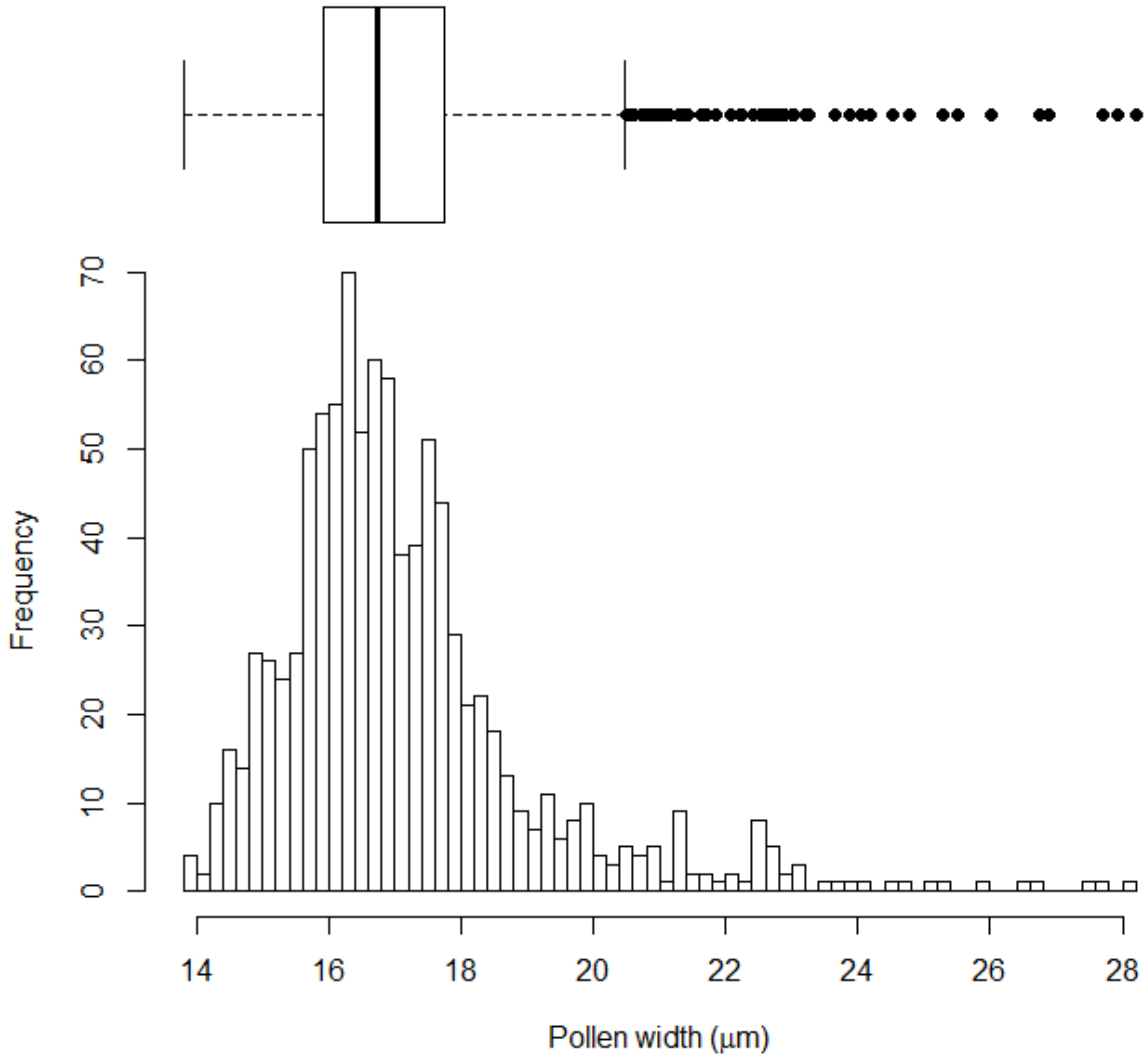


Figure 1.5: Distribution of pollen grain widths for staminate two hybrids, as represented by a histogram and boxplot. Pollen grains for both parental species are typically 18-23 µm wide.



1.4 Discussion

In the survey of *Celastrus* individuals across the eastern USA, only 4.2% were hybrids (20 of 475 genotypes). When excluding *C. scandens*, the proportion of invasive individuals that were hybrids was 8.0%. Therefore, the hybrids themselves are not the driving force behind the invasion by *C. orbiculatus*. Distances between sites with the rare hybrids were generally quite large, ranging from the southern Great Lakes region, New England, and the southern Appalachian Mountains (Table III), indicating that independent hybridization events occurred throughout the range of sympatry. Although established hybrids are uncommon, other evidence in our study suggests that gene flow and reproductive interference may be important for declining populations of *C. scandens*. Unidirectional pollen flow is evident from genetic analysis of maternally inherited markers, where all 20 hybrids found in this study showed the cpDNA signature of *C. scandens*. When asymmetry in pollen or gene flow favors males in an introduced species, declines in a native species are hastened (Prentis et al. 2007).

Hybridization rates here are low compared to studies of hybrid swarms. For example, Ayres et al. (2008) found over 90% of cordgrass seeds in the marshes they studied were from the invasive hybrid *Spartina foliosa* x *alterniflora*. Zalapa et al. (2009) found that more than half of the individuals sampled in a contact zone between *Ulmus rubra* and *U. pumila* were hybrids, while Hoban et al. (2009) determined that one-third of individuals at their study sites were hybrids between *Juglans cinerea* and *J. ailantifolia* individuals. For *Celastrus*, hybrids are found throughout the range of sympatry between the two parental species (Table III), but there is no evidence of a “hybrid swarm” or that invasiveness evolved after interspecific hybridization. Unlike other studies that found hybrids to be more fit than the native parental species or that hybrids effectively excluded the native parental species (Vila and D'Antonio 1998, Burgess and Husband 2006, Moody and Les 2007), the hybrids we studied showed signs of reduced fitness due to reduced fecundity. However, the sheer number of flowers produced by hybrids is quite large compared to *C. scandens* (*pers. obs.*). Like *C. orbiculatus*, the axillary inflorescences

present all along a stem in hybrids (Fig. 1.2) allow for greater floral and fruit production, which is in contrast to the terminal panicles of *C. scandens* that mark the end of a stem (Steward et al. 2003). It is possible that the large floral output in the hybrid, as well as an increased growth rate and maximum size (Pooler et al. 2002), largely compensates for the disadvantage in reproductive efficiency compared to *C. scandens*, although I did not explicitly test this possibility. Additionally, backcross and later-generation hybrids can in some cases regain complete fertility (Rieseberg 2000, Hegarty et al. 2009). A future role of hybrid individuals as invasive competitors cannot be ruled out as the ongoing invasion by *C. orbiculatus* in North America is relatively new and transitions in the frequency of hybrids can be initially slow, even in cases where hybrids finally dominate (Ferdy and Austerlitz 2002). Also, a distinction must be made between intra- and interspecific hybridization. The results presented here do not exclude the possible role of intraspecific hybridization and novel genotype combinations leading to increased invasiveness in *C. orbiculatus*, which has been reported elsewhere for other invasive species (Ellstrand and Schierenbeck 2000, Durand et al. 2002, Kolbe et al. 2004, Lavergne and Molofsky 2007).

Unidirectional pollen flow during invasion can threaten native species through two main mechanisms. Introgression can lead to the loss of genetically pure individuals, and fitness can be reduced as limited female reproductive effort is disproportionately wasted on hybrid individuals. Previous research on hybridizing introduced plants has forecasted rapid declines in the future of native species as a result of unidirectional pollen flow (Prentis et al. 2007). Other researchers have found “pure” lines of native species being lost to introgression (e.g. Rhymer et al. 1994). High selfing rates may help prevent introgression and population declines due to hybridization (Wolf et al. 2001), but *C. scandens* is almost always dioecious, and thus is incapable of self-fertilization. The extent of the threat to *C. scandens* through hybridization and reproductive interference cannot be determined through a survey of established plants across the species range, as it does not provide input on the proportion of reproductive effort devoted

to hybrid offspring. The magnitude of reproductive decline due to interspecific gene flow can be better assessed when directly studying the offspring or seed crop of progenitor individuals (e.g. Barbour et al. 2002, Prentis et al. 2007, Burgess et al. 2008). Additionally, the mechanism leading to unidirectional pollen flow is currently unknown. Unidirectional gene flow may arise due to differences in ploidy, but is not a likely explanation for *Celastrus* as previous work has shown both species to have the same number of chromosomes, 2n = 46 (White and Bowden 1947). Other mechanisms that can cause unidirectional pollen flow include a numerical advantage for one of the parental taxa (Burgess et al. 2005), differences in male fecundity, or differences in the ability to recognize and reject interspecific pollen (Anttila et al. 1998).

How important is interspecific gene flow for *Celastrus* in North America? Hybridization does not appear to have played an important role in the rapid spread of *C. orbiculatus* across its introduced range in the last century. Conversely, it is quite possible that interspecific pollen flow has been involved in the decline of *C. scandens*. There are likely other factors at play in the decline of *C. scandens*, including habitat loss and superior performance by *C. orbiculatus* as a competitor (Dreyer et al. 1987, Leicht-Young et al. 2007b, Ashton and Lerdau 2008). However, multiple factors often contribute to widespread species decline (Didham et al. 2007). Additionally, reproductive interference is a much more efficient mechanism of species exclusion than competition (Kishi et al. 2009). Reproductive interference is a plausible culprit in the decline of *C. scandens* considering the vitality of other native lianas in eastern North America.

For land management and conservation efforts, there are two clear messages. Hybrid *Celastrus* exists in the wild, and its identification in the field based on morphological characteristics will be problematic. It is difficult to study hybridization without genetically inherited molecular markers (Allendorf et al. 2001), and the problem is exacerbated with *Celastrus* because the parental species are morphologically very similar before maturation and development of reproductive structures (Leicht-Young et al. 2007a). However, if trying to control invasion, culling of hybrids does not need to be of primary concern because they are (for

the time being) relatively rare. Of more immediate concern is protecting *C. scandens* from reproductive interference. *Celastrus orbiculatus* is usurping ovules of *C. scandens* (Burgess et al. 2008), which means reduced fertility and perhaps the eventual loss of genetically pure lines of *C. scandens*. Reproductive interference places a premium on the removal of the introduced liana where the native species is present during the early stages of invasion, before staminate individuals reach reproductive maturity—although that is the stage in which identification is most difficult. Future research of *Celastrus* that concentrates on hybridization at a finer scale, investigating the genetic identity of seeds collected directly from maternal plants, would be useful in quantifying the degree to which *C. orbiculatus* interferes with *C. scandens* reproduction, and the causes of asymmetric hybridization.

The work presented here supports the claim that species introductions can threaten native biodiversity, especially when coupling a closely related native and introduced species. My findings also contribute to a growing body of work pointing to negative effects of invasion that arise due to processes (interspecific gene flow) that are often overlooked in favor of other commonly investigated mechanisms, such as competition and predation.

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2. REDUCED REPRODUCTIVE SUCCESS IN A NATIVE VINE DUE TO REPRODUCTIVE INTERFERENCE FROM AN INTRODUCED CONGENER

2.1 Introduction

Studies of the interaction between introduced species and closely related native species traditionally concentrate on the role of resource competition (Mack 1996). Increasingly, an appreciation for the role of reproductive interference, such as hybridization, during invasion has developed (Rhymer and Simberloff 1996). Empirical and theoretical work has shown that hybridization associated with species introductions can accelerate declines of native species (Wolf et al. 2001). Reproductive interference and hybridization can be even more detrimental to native species than resource competition. Reproductive interactions are especially harmful because they display positive density dependence, whereas resource competition follows a negative density dependence pattern (Groning and Hochkirch 2008). Additionally, in sessile organisms, the physical scale over which reproductive interactions (pollinations in plants) occur can far exceed the scale of competitive interactions (Takakura et al. 2011, Nishida et al. 2012). Finally, reproductive interactions can intensely target a single or small set of species, whereas the effects of resource competition are scattered across an ecological community.

In plants, interspecific pollen flow can lead to declines in populations through a number of mechanisms. Reproductive interference can occur when pollen from a closely related species prevents successful fertilization by conspecific pollen (Matsumoto et al. 2010). Female reproductive effort may be wasted on fertilized ovules that rarely develop into viable seeds (Prentis et al. 2007), and hybridization may lead to the decline of a species due to seed discounting and wasted reproductive effort (Burgess et al. 2008). Additionally, hybrids may be especially numerous, fit, or fecund, leading to “hybrid swarms” that exclude one or both parental species (Hall et al. 2006, Ayres et al. 2008).

Advantages in resource competition may be counteracted by reproductive interference when the interaction between two species is asymmetric (Kishi et al. 2009). Asymmetry in gene flow or reproductive interference may arise due to differences in abundance of the parental taxa (Wirtz 1999, Burgess et al. 2005), differences in male fecundity, and differences in the ability to exclude heterospecific male gametes (Anttila et al. 1998). When asymmetry in pollen or gene flow favors male fecundity in an introduced species or subspecies, declines in the native taxon are hastened (Buggs and Pannell 2006).

The introduction of non-native *Celastrus orbiculatus* to North America, and its subsequent spread across the native range of *C. scandens*, provides an opportunity to study the reproductive interaction between an invasive and declining native species. In the USA, *C. orbiculatus* has spread over much of the range of *C. scandens*. The two species are known to hybridize in controlled settings (Pooler et al. 2002) and in the wild (see Chapter 1). While hybrid individuals in the wild are not common, strong asymmetry in hybridization is evident. All hybrid individuals found in wild populations had a *C. scandens* maternal lineage (Chapter 1). Although asymmetric hybridization in the wild has been established, the *rate* of hybridization and *degree* of asymmetry, and whether it is linked to the decline of *C. scandens* observed in parts of its range (Steward et al. 2003, Leicht 2005), remain unknown. While a survey of plants across a large geographical range can provide some insight into the dynamics of invasion and hybridization, direct studies of offspring or seed crops of progenitor individuals are required to confidently determine the magnitude of reproductive decline due to interspecific gene flow (e.g. Prentis et al. 2007). Additionally, studies across broad geographical ranges provide little to no insight into the underlying processes leading to asymmetrical gene flow.

At the Indiana Dunes National Lakeshore, *C. scandens* and *C. orbiculatus* have similar abundances, providing an ideal opportunity to investigate reproductive interactions. I evaluated the effect of proximity and floral output of conspecific and interspecific males on fertilization and hybridization rates in both species to elucidate the mechanisms underlying asymmetrical pollen

flow. I used field observations to determine: 1) whether there is a correlation between fertilization rate and availability of conspecific and interspecific pollen; 2) whether the two species differ in their response to pollen availability, as a negative relationship between fertilization and interspecific pollen availability would be consistent with prezygotic reproductive interference; 3) the rate of hybridization in pistillate plants of both species and the degree of asymmetry, 4) the correlation between hybridization rate and availability of conspecific or interspecific pollen for *C. scandens*, and 5) male fecundity (staminate flower output) in both species, where a large difference in male fecundity may lead to pollen swamping and asymmetric gene flow. Also, I used manipulative hand-crosses to compare the likelihood that pistillate plants of *C. scandens* and *C. orbiculatus* will be fertilized by interspecific pollen.

This study will help assess the extent of reproductive interference between *C. orbiculatus* and *C. scandens*, give insight into causes of asymmetrical gene flow, determine the magnitude of threat that hybridization poses to *C. scandens* reproduction, and provide insight in how to best conserve populations of the increasingly rare native plant.

2.2 Methods

2.2.1 Study species

Celastrus scandens L. (Celastraceae), commonly known as American bittersweet or American staff vine, is the only member of the genus native to North America (Hou 1955). It is a liana (woody vine) usually found in open habitat. Its range extends from southern Quebec to South Dakota, south to western Texas through Georgia. The native range of *C. orbiculatus* Thunb., commonly known as oriental or Asiatic bittersweet, is in far eastern Asia, in Korea, Japan, and China (Hou 1955). It is found in thickets and lowland slopes, but can thrive in shaded habitat that would likely exclude *C. scandens* (Pavlovic and Leicht-Young 2011). Both species are usually dioecious, although rare individuals and populations displaying other breeding systems are known. Insects, particularly native bees, pollinate both species, and birds

are largely credited as the dispersers of the seeds that sit inside bright red fleshy fruits surrounded by an orange (*C. scandens*) or yellow (*C. orbiculatus*) capsule (Brizicky 1964).

Celastrus orbiculatus was introduced as an ornamental vine to the eastern USA in the mid- to late-nineteenth century. The first reports of it escaping into the wild came in the early twentieth century; by the middle of the twentieth century it was widely recognized as a pest species rapidly spreading in the eastern USA (Patterson 1974). Land managers and foresters consider *C. orbiculatus* a troublesome species because it is a strong competitor that crowds out native vegetation, negatively affects forestry operation, and can alter natural successional trajectories (Fike and Niering 1999, Leicht-Young et al. 2007b). Notably, *C. scandens* has declined most in areas where invasion by *C. orbiculatus* is oldest and most extreme (Dreyer et al. 1987, Stewart et al. 2003, Leicht 2005, R. I. Bertin *pers. comm.*), although it is not clear whether or not there is a direct relationship between the two patterns. *Celastrus scandens* has been listed as a species of special concern in several states at the center of its range; in Delaware it may be extirpated, while it is listed as a vulnerable species in New York, a threatened species in Massachusetts, and an endangered species in North Carolina.

2.2.2 Study site and sampling

The field observations and manipulative crosses took place at the Portage Lakefront within the Indiana Dunes National Lakeshore (near 41.63^{O} N, 87.18 ^{O} W). The study site is composed of southern Lake Michigan sand dunes and wooded edges, bound by the town of Ogden Dunes, Indiana to the west and the Port of Indiana to the east (Fig. 2.1). The site was chosen because reproductive individuals of both species are abundant, which is uncommon. I attempted to include all flowering individuals in the observational studies, but some flowering individuals may have been in nearby residential areas, off of National Park Service property, and were not included in the study. In total, the study included 39 *C. orbiculatus* individuals (22 staminate, 14 pistillate, and 3 putatively monoecious) and 40 *C. scandens* individuals (17 staminate, 21 pistillate, and 2 putatively monoecious).

Figure. 2.1: Spatial distribution of focal *Celastrus* individuals at the Protage Lakefront study site.

Field identification using reproductive structures (Leicht-Young et al. 2007a) was verified by genetic analysis of leaf tissue (see Chapter 1). Each individual was scored at five microsatellite loci, and genetic identities were tested using two Bayesian clustering approaches, STRUCTURE version 2.3 and NewHybrids version 1.1 beta (Anderson and Thompson 2002, Falush et al. 2007). Spatial coordinates were measured with GPS for each individual included in the study. The coordinates were used to calculate the distance to each staminate plant for every pistillate individual.

2.2.3 Male floral output

I recorded the total number of open staminate flowers for each individual on 15 dates, between 20 May and 12 June 2008. All the open flowers on an individual were counted and recorded when possible. Some individuals were too large or climbed too high to count all flowers, in which case a proportional sampling technique was used. I counted the number of flowers for a smaller randomly selected area, and the total floral output was estimated by extrapolation to the full area covered by an individual.

2.2.4 Manipulative hand-crosses

Manipulative hand-crosses were carried out in 2006 to test differences between the species in accepting interspecific pollen, a possible mechanism behind asymmetric hybridization. Inflorescences on five pistillate plants of each species were bagged with bridal mesh while still in bud, to prevent pollination. For each pistillate plant, 25 flowers were hand-pollinated with freshly dehisced anthers from five staminate plants of the other species (five flowers were devoted to each staminate plant). In total, 250 flowers were hand-pollinated. The flowers were observed approximately 10 days later for signs of fertilization.

2.2.5 Comparing fertilization and hybridization rate between species

Every date plants were observed, I marked up to 10 newly opened flowers on each pistillate plant with colored string tied to the pedicel. Flowers were chosen haphazardly, as attempts to randomize flower selection were prohibitively slow. I checked each flower for

evidence of fertilization 9 to 17 days after it was marked, and followed the development of these flowers through fruit maturation. The flowers on the cyme of *C. orbiculatus* are all of equal rank, while the inflorescence for *C. scandens* is a panicle with a hierarchical structure. The position on the inflorescence was recorded for each marked *C. scandens* flower in order to investigate potential differences in maternal investment and fertilization rate (see Wesselingh 2007). On 5 October and 11 October 2008, the fruits that resulted from the marked flowers were collected. The resulting seeds were prepared for germination according to the method described in Young and Young (1992), and sowed in the greenhouse at the University of Illinois at Chicago after 90 days of cold stratification. In total, 104 seedlings resulted from the original 716 flowers marked (43 seedlings from 317 *C. scandens* flowers, and 61 seedlings from 399 *C. orbiculatus* flowers). I genetically tested the seedlings to determine the species of the pollen donor using the nuclear microsatellite DNA markers and statistical methods described in Chapter 1. Microsatellite markers are an especially useful tool in studies investigating pollination patterns of wild plants (Ashley 2010). I tested the relationship between fertilization rate and a number of predictors, including species identity and the availability of interspecific and conspecific pollen. The sample size of total seedlings was too small to test correlations with multiple predictors, but the difference between species was tested. The details of the data analyses are described below.

2.2.6 Predictors of hybridization rate in *C. scandens*

The fruits collected during the fertilization study were supplanted by additional fruit collections from *C. scandens*, with the goal of increasing the sample size and statistical power. Emphasis was placed on fruits from the native species because a) no wild hybrids found had a *C. orbiculatus* maternal lineage (see Chapter 1), and b) there is more urgency in understanding hybridization in *C. scandens*, as it is species of concern in much of its native range. Fruits were collected from 18 *C. scandens* pistillate plants. Again, I tested the relationship between hybridization rate and a number of predictors relating to the availability of interspecific and conspecific pollen (detailed below).

2.2.7 Data analysis

To test the hypothesis that fertilization rate in wild *C. scandens* and *C. orbiculatus* differed, and that the two species differed in their response to conspecific and interspecific pollen availability, I used generalized linear mixed models (GLMM), with maternal identity as a random factor. The subjects of the analysis were individual flowers (n = 716, in 40 maternal groups). The response variable tested was the binary fate of a flower at the fertilization stage (fertilized or unfertilized). Three explanatory variables, and all possible interactions, were tested as fixed effects: species identity, weighted conspecific mating potential, and weighted interspecific mating potential. The weighted mating potential was calculated after Wagenius et al. (2007), with some modification.

2.2.7.1 Equation 1:

$$P_i = \sum_{j=1}^{n} e^{-\gamma d_{ij}} \log_{10}\left(F_j\right)$$

I removed an incompatibility term, c_{ij}, and instead excluded other pistillate plants in the calculation (those which would have $c_{ij} = 0$). I weighted the mating potential by the $\log_{10}$ male floral output, F_j, for each *j*-th father on the particular day that a flower was marked. The distance from the *i*-th mother (which carried the flower) to each *j*-th father is d_{ij}. I used the same value for γ, the inverse of the mean pollination distance (13.3 m^{-1}), as Wagenius et al. (2007), as they also studied an insect-pollinated self-incompatible plant (*Celastrus* spp. are dioecious).

To test for differences in hybridization rate between the two species, I used a generalized linear model (GLM) with a binomial response. The explanatory variable was species identity of maternal plant, and the subjects were the individual maternal plants (n = 19 [nine *C. scandens* and 10 *C. orbiculatus*], with 104 total seedlings). The GLM was also used to examine the results of interspecific hand-crosses, where fertilization success in interspecific

crosses was the response and species identity of maternal plant was the explanatory variable (n = 10 pistillate plants, five of each species).

To test the relationship between hybridization rate in *C. scandens* and the availability of conspecific and interspecific pollen, I again used a GLM with binomial response. The subjects of the analysis were individual maternal plants from which seeds were collected and germinated (n = 18, with 259 total seedlings). The response variable was the proportion of successfully germinated seedlings that were hybrids. Four factors, and all two-way interactions, were included in the model: distance to the nearest conspecific male (staminate *C. scandens*), distance to the nearest interspecific male (staminate *C. orbiculatus*), the $\log_{10}$ floral output of the nearest co-flowering conspecific male, and $\log_{10}$ floral output on the nearest co-flowering interspecific male. A staminate plant was considered as "co-flowering" if it had open staminate flowers on the mean flowering date of the pistillate subject.

All statistical tests were completed using R version 2.15.1 (R Core Team 2012). The GLMM tests were implemented using the *lme4* package, with the maximum number of evaluations (*maxFN*) and iterations (*maxIter*) during optimization both set to 10,000. When using a GLM or GLMM, I performed model selection by starting with a maximal model and proceeding through stepwise backward elimination. The largest interaction terms were removed from the model first, and the factor or interaction to be eliminated next was determined with a random number generator (Crawley 2005). I did not attempt to remove factors from the model if they were included in a significant interaction. A cutoff of $\alpha = 0.05$ was used to determine whether or not to eliminate a factor.

2.3 Results

2.3.1 Staminate floral output and hand-crosses

Data from the observations of staminate floral output and manipulative hand-crosses both show a significant advantage for male fecundity in *C. orbiculatus*. Staminate flowering in

C. orbiculatus lasted 21 days, from 20 May to 9 Jun, while in *C. scandens* staminate flowering lasted 16 days, from 28 May to 12 Jun (Fig. 2.2). The peak flowering dates were offset by 5 days—31 May for *C. orbiculatus*, 5 Jun for *C. scandens*. There was a 200-fold difference in the floral output per staminate individual. Even on the peak flowering date for *C. scandens* there were 77 times more *C. orbiculatus* flowers per staminate individual.

In the manipulative hand-crosses, *C. scandens* was nearly 20 times more likely to be fertilized by *C. orbiculatus* pollen than vice versa (41% in *C. scandens* [s.e. = 4.4%] and 2.4% in *C. orbiculatus* [s.e. = 1.4%]; n = 5 maternal plants with 125 flowers in both species). The difference between the species was significant (GLM, $p < 0.01$).

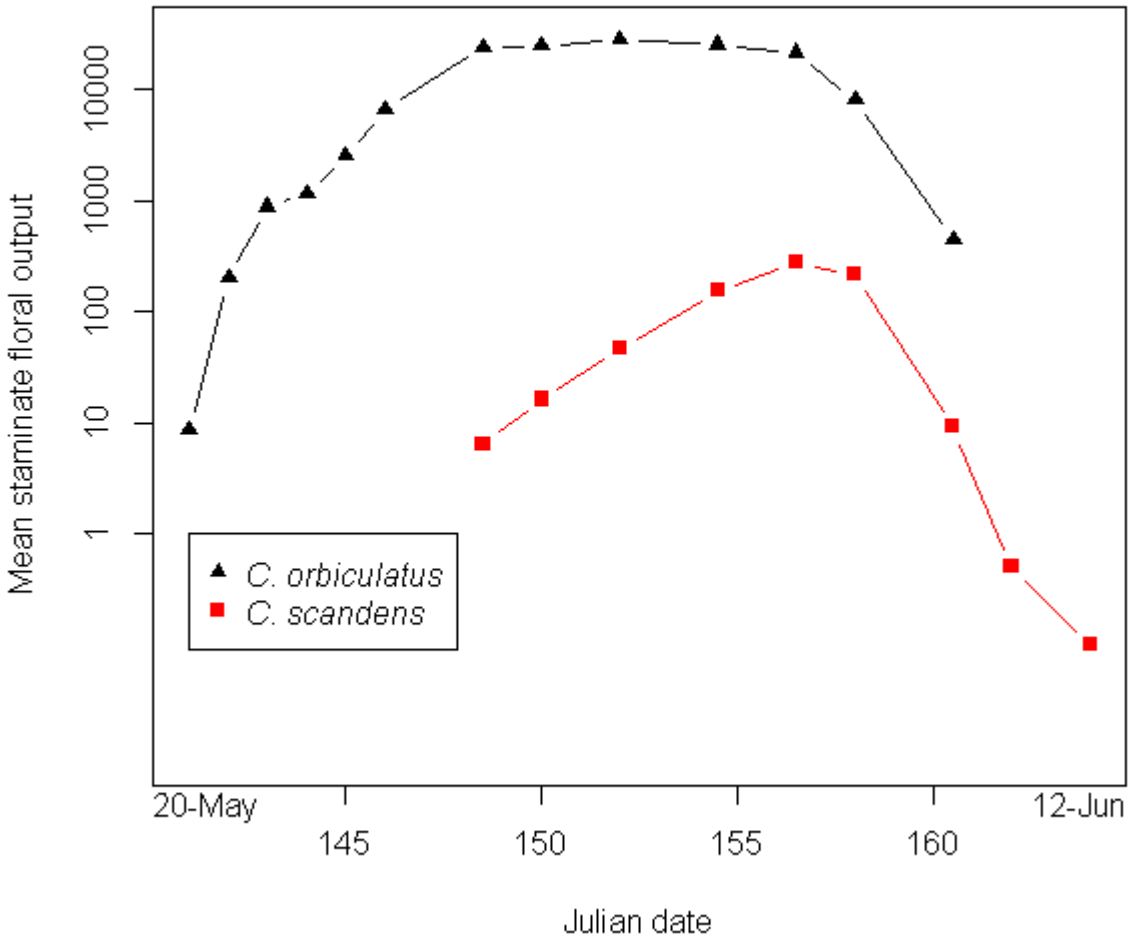
2.3.2 Fertilization rate in *C. scandens* and *C. orbiculatus*

Fertilization rate was 27.4% for *C. scandens* (s.e. = 2.9%, n = 317) and 44.1% for *C. orbiculatus* (s.e. = 3.3%, n = 399). Weighted conspecific mating potential was significantly, and similarly, positively correlated with fertilization in both species (GLMM, $p < 0.01$). There was a significant interaction between species identity and weighted interspecific mating potential ($p < 0.02$). In *C. orbiculatus*, fertilized and unfertilized flowers were similar with respect to the interspecific mating potential at the date on which they opened. In *C. scandens*, fertilized flowers had substantially greater interspecific mating potential. Both species were more likely to have greater fertilization rates with increased conspecific mating potential. Only *C. scandens* had a greater fertilization rate with increased interspecific mating potential. Analyses of *C. scandens* flower fate showed that inflorescence position was not significantly correlated with fertilization success (generalized linear mixed model, with maternal plant as random factor; $p > 0.5$), thus inflorescence position was excluded from all further analyses.

2.3.3 Hybridization rate in *C. scandens* and *C. orbiculatus*

Analyses of germinated seedlings that arose from flowers marked in the fertilization study showed a huge and significant difference in the number of hybrid seedlings between the two species (GLM, $p < 0.001$). In *C. scandens*, 51% of seedlings that germinated were hybrids

Figure 2.2: Mean staminate floral output per day. For *C. scandens*, n = 19 (red squares) and *C. orbiculatus* n = 25 (black triangles).



(n = 9 maternal plants, with 43 total seedlings; s.e. = 11.4%), while only one of the 61 (1.6%) *C. orbiculatus* seedlings was a hybrid.

2.3.4 Predictors of hybridization rate in *C. scandens*

When an additional 216 *C. scandens* seedlings were included, which were not part of the fertilization portion of this study, the model for best predicting hybridization rate included the distance to the nearest staminate *C. orbiculatus*, log floral output of the nearest co-flowering staminate *C. orbiculatus*, and the interaction of these two predictors (GLM, $p < 0.02$). The distance to ($p > 0.5$) or floral output ($p > 0.18$) of the nearest conspecific male did not significantly predict hybridization rate in *C. scandens* pistillate plants. Plants that were nearest to *C. orbiculatus* staminate plants had the greatest expected hybridization rate (Fig. 2.3). Floral output of nearby interspecific males was negatively correlated with hybridization rate, and that correlation was most pronounced in pistillate individuals furthest from interspecific males (Fig. 2.4).

2.4 Discussion

The results of this study provide strong evidence that reproductive success in *C. scandens* is altered by *C. orbiculatus*. There is no evidence to support the reverse interaction. Fertilization rates were greater in *C. orbiculatus*, but it does not appear as though the difference is due to obstruction of *C. scandens* fertilization by *C. orbiculatus* pollen. Observational data on fertilization rates in both species suggest that the availability of conspecific staminate flowers increases fertilization success (Fig 2.2). The relationship between fertilization success and interspecific mating potential—an index that combines the inverse distance of the nearest interspecific male and its $\log_{10}$ floral output—differs between the two species. Fertilized and unfertilized flowers in *C. orbiculatus* had similar weighted interspecific mating potentials, suggesting that fertilization in *C. orbiculatus* is not correlated with proximity to or floral output of

Figure 2.3: Relationship between hybridization rate and distance to the nearest interspecific male in *C. scandens*. These data are from unmanipulated pistillate plants. The curve represents the predicted values from a GLM with binomial response (n = 18 pistillate plants, with 259 total seedlings).

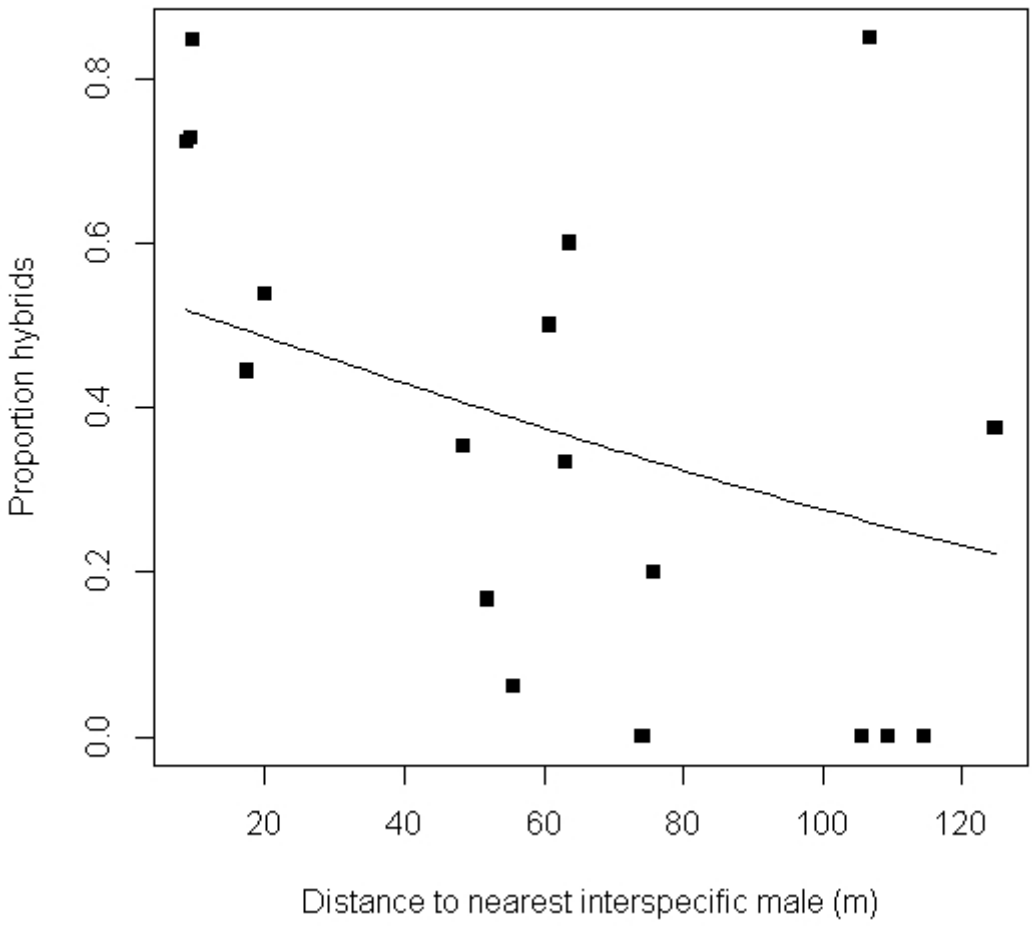
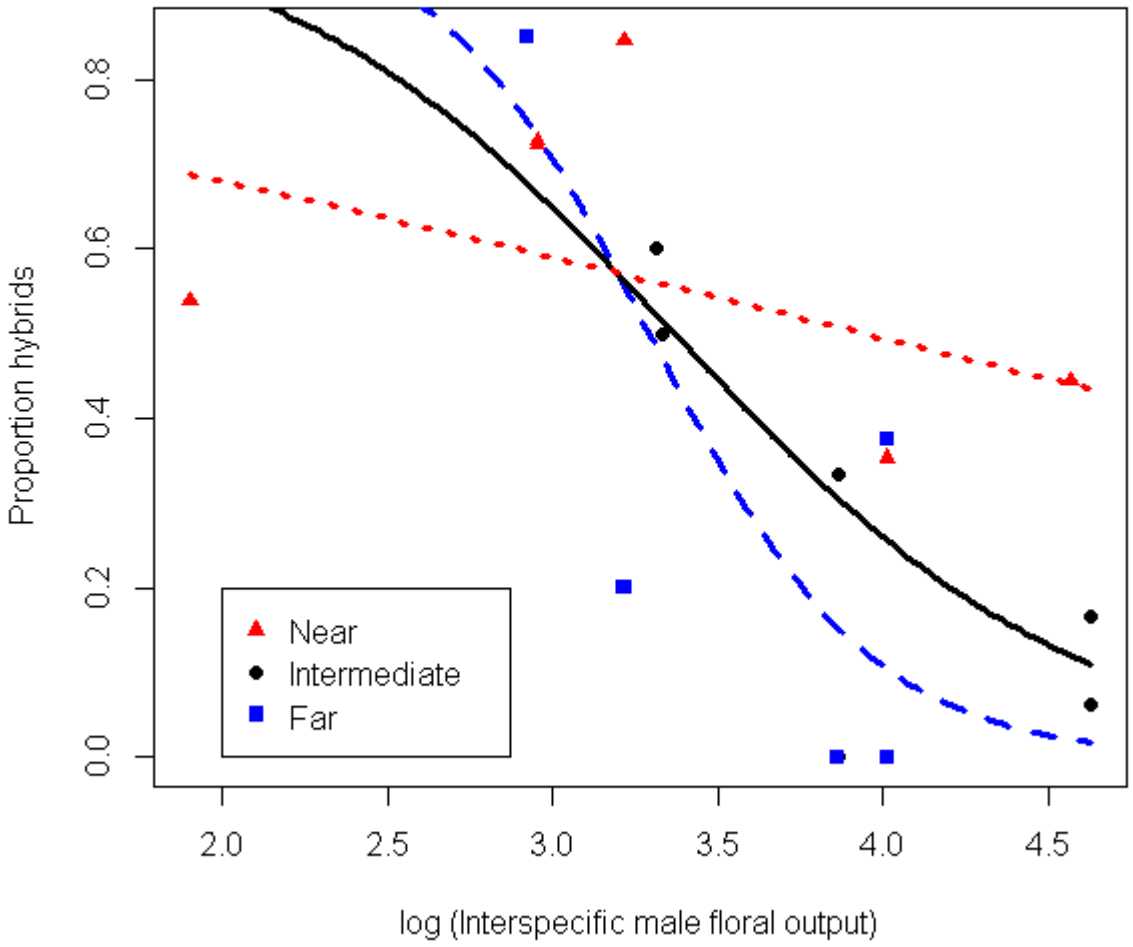


Figure 2.4: Hybridization rate in *C. scandens* versus the interaction of distance to the nearest interspecific male and $\log_{10}$ floral output of the nearest co-flowering interspecific male. Each point in this figure is a single pistillate *C. scandens* individual (n = 18). Red triangles represent the six pistillate individuals that were nearest to a C. orbiculatus staminate plant, blue squares represent the six pistillate individuals that were furthest, and black circles represent the six pistillate individuals of intermediate distance. Each curve shows the predicted hybridization rates for individuals in the distance class that corresponds to the curve's color. GLMM showed the interaction was a significant predictor of hybridization rate in *C. scandens* ($p < 0.02$).



C. scandens pistillate plants. Conversely, fertilized flowers on *C. scandens* pistillate plants had substantially greater weighted interspecific mating potential ($p < 0.02$). Proximity to flowering staminate *C. orbiculatus* is correlated with increased fertilization in *C. scandens*. This is contrary to what one would expect if interspecific pollen interferes with successful fertilization of *C. scandens*, as has been observed elsewhere (Takakura et al. 2009). The enhanced fertilization rate in *C. scandens* suggests increased fertilization due to release from pollen limitation, or superior pollen viability in *C. orbiculatus*.

Genetic tests of seedlings show results consistent with the observations from the fertilization stage of the study. The hybridization rate in *C. scandens* was 51%, while it was only 1.6% in *C. orbiculatus*. The levels of hybridization are notable because of the extreme asymmetry (nearly unidirectional) and because a majority of *C. scandens* seedlings studied here resulted from interspecific crosses. If hybrid seedlings are not considered as contributors to fitness, it is plausible that the decline in *C. scandens* in the presence of *C. orbiculatus* can be tied to the reproductive effort or opportunity wasted on hybrids.

Hybridization rate in *C. scandens* was, not surprisingly, negatively associated with distance to the nearest interspecific individual (Fig. 2.3). All individuals within 30m of a staminate *C. orbiculatus* individual had a hybridization rate greater than 40%. Of the five individuals more than 100m from the nearest *C. orbiculatus*, only one had a hybridization rate greater than 40%. There was a significant interaction between distance to the nearest staminate *C. orbiculatus* and log floral output of the nearest flowering staminate *C. orbiculatus* (Fig. 2.4). The relationship between hybridization rate and floral output was the opposite of what I initially expected. In plants near staminate *C. orbiculatus*, the predicted hybridization rate remained high in the model, regardless of the floral output. However, for plants further from *C. orbiculatus* staminate individuals, hybridization rate *decreased* with increasing male floral output. This is counterintuitive, as I expected a greater reproductive footprint of males with many flowers. However, the negative relationship might be explained by pollinator behavior,

where large floral output may enhance within-individual pollinator visits (analogous to geitonogamy in cosexual plants), thus reducing pollination success (Karron and Mitchell 2012). Alternatively, there may be a confounding factor that was not addressed in this portion of the study, as it was strictly observational. Interestingly, the proximity or floral output of conspecific males did not significantly correlate with hybridization rate in *C. scandens*.

Results from observation and experimental portions of this study provide evidence that two mechanisms may be simultaneously acting to create asymmetric hybridization in *Celastrus*. Despite the approximately equal abundance of plants carrying staminate flowers at the study site (n = 25 and 19 for *C. orbiculatus* and *C. scandens*, respectively), there was a 250-fold advantage for *C. orbiculatus* in terms of total floral output (Fig. 2.2). The extreme discrepancy in fecundity is likely due in part to the generally enhanced growth of introduced *C. orbiculatus* individuals (Leicht-Young et al. 2011), as well as the axillary positioning of inflorescences in *C. orbiculatus* that allows flowers to form along the whole stem—as opposed to the terminal inflorescences in *C. scandens* that are limited to the end of a stem (Steward et al. 2003). Additionally, these results may underestimate the male advantage of *C. orbiculatus*, as pollen viability in the introduced species can be greater (Dreyer et al. 1987). The increased male fecundity likely leads to “pollen swamping”, and could cause *C. orbiculatus* to negatively impact *C. scandens* reproduction even when the introduced species is found in low densities. Meanwhile, the high male fecundity of *C. orbiculatus* may explain the increased fertilization rate in the corresponding pistillate plants, as pollen limitation may be reduced.

Experimental hand-crosses showed that *C. scandens* readily accepts *C. orbiculatus* pollen, but *C. orbiculatus* rejects pollen from *C. scandens* in a vast majority of cases, providing direct evidence that the two species react differently when interspecific pollen arrives to a stigma. Differences in prezygotic barriers can be important in the creation of asymmetric hybridization (Rhymer and Simberloff 1996). This difference in the ability to recognize and reject interspecific pollen might be expected in this system; the native range of *C. orbiculatus*

overlaps with several congeners (Hou 1955), while *C. scandens* is not naturally sympatric with any closely related species (Noor 1997).

The positive relationship between the availability of interspecific pollen and fertilization rate observed in *C. scandens* ran counter to the expectations of reproductive interference that acts prezygotically, such as that observed by Takakura et al. (2011) in *Taraxacum*. However, increased fertilization in the presence of a closely related conspecific is not unique. Anttila et al. (1998) found that the pollen of *Spartina alterniflora*, a cordgrass introduced to the California coast, increased seed set in the native *S. foliosa*, while *S. foliosa* pollen did not affect seed set in *S. alterniflora*. The scale at which reproductive interference acts on *C. scandens* is large. The best model predicts a hybridization rate of 40% for *C. scandens* that were 50m from the nearest *C. orbiculatus* staminate plant, and 27% for individuals that were 100m away (Fig. 2.3). My results are comparable to Burgess et al. (2008), who studied introduced white (*Morus alba*) and native red (*M. rubra*) mulberries in southern Ontario. After removing all introduced individuals and hybrids within 50m of the native individuals, they found 63% of seedlings collected from *M. rubra* were fertilized by the *M. alba* or hybrids. The scale of reproductive interference in *Celastrus* is much larger than the range found by Takakura et al. (2011) for a native (*Taraxacum japonicum*) and introduced dandelion (*T. officinale*) near Osaka, Japan, although they conducted their study at a much smaller spatial scale. The proportion of hybrid seedlings I found in *C. scandens* (51%) is large compared to some other studies, such as the 16% and less reported for *Eucalyptus nitens* and *E. ovata* in Tasmania (Barbour et al. 2002) or the 30% and less for *Eucalyptus benthamii* and *E. viminalis* in New South Wales (Butcher et al. 2005), but it is not unprecedented. In unmanipulated plots, Burgess et al. (2008) found 77% of seedlings from by *M. rubra* were hybrids. Buggs and Pannell (2006) found a high degree of asymmetry in hybridization between diploid and hexaploid populations of *Mercurialis annua*, where experimental plots resulted in 20-80% hybrid seedlings from hexaploid individuals. When measured per individual, the 200-fold discrepancy in male fecundity between *C. scandens* and

C. orbiculatus is extreme when compared to other studies described above. Thus, asymmetric hybridization is likely caused both by differences in male fecundity and rejection of interspecific pollen (Anttila et al. 1998).

Overall, the results of the observational and manipulative studies strongly suggest that the decline of *C. scandens* in its historical range is partly due to reproductive interference by *C. orbiculatus*. Although *C. orbiculatus* has been shown to be a superior competitor in several respects and can escape the negative effects of high density (Dreyer et al. 1987, Ashton and Lerdau 2008, Leicht-Young et al. 2011), the targeted nature of reproductive interference, the rapid declines of native taxa associated with it, and the large area over which it can act make it a more likely explanation than resource competition alone. It is unlikely that the *Celastrus* system has reached equilibrium, as *C. orbiculatus* is a recent invader of North America that continues to spread and proliferate. The positive density-dependence associated with reproductive interference suggests that the decline of *C. scandens* will accelerate and spread over a larger area, unless large and broad efforts to reverse the spread of *C. orbiculatus* are undertaken. A feedback between decreased abundance and increased hybridization in *C. scandens* may occur (Levin et al. 1996), potentially leading to extirpation from much of its native range.

Efforts to conserve *C. scandens* can derive some guidance from my results. Reducing exposure to *C. orbiculatus* must be a priority. The degree of isolation from *C. orbiculatus* must be greater than conventional cases where only competition is considered, as reproductive interference in this system can be substantial at distances as great as 100m. Previous work (Chapter 1) showed that hybrids are not common participants in the invasion of North America, and show signs of greatly reduced fecundity. As a result, the loss of genetic identity of *C. scandens* due to introgression has not yet occurred. Thus, *C. scandens* populations that were previously exposed to reproductive *C. orbiculatus* individuals may still represent pure lines that are suitable for conservation or re-establishment. Additionally, closer inspection of *C.*

scandens populations may be warranted throughout its range. In many eastern states, *C. scandens* is now absent from many sites that it was documented historically (S. Leicht-Young *pers. comm.*). It is possible that the decline of this species is more widespread and severe than previously thought, and the current situation may warrant protected status over a greater portion of the native liana's range.

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3. MISLABELING OF AN INVASIVE VINE (*CELASTRUS ORBICULATUS*) AS A NATIVE PLANT (*C. SCANDENS*) IN HORTICULTURE

3.1 Introduction

Invasive plant species are costly to human economies and harmful to natural environments. Rapidly spreading introduced plant species, or invasive species, in the USA cause over $25 billion-a-year in losses and damages, not including the costs of control and removal (Pimentel et al. 2005). Additionally, invasive species disturb ecosystems and natural processes, such as succession and nutrient cycling (Ehrenfeld 2003, Rudgers et al. 2007). Many introductions have been the direct result of human cultivation, be it agriculture or horticulture. About half of the invasive plants in the USA were deliberately introduced (Mack and Erneberg 2002), and 85% of invasive woody plants were first introduced as ornamentals (Reichard and Hamilton 1997).

There has been an increasing emphasis on using native plants as ornamentals, in part because horticulturalists increasingly recognize the problem of invasive plants (Peters et al. 2006). Consumers are encouraged to use native plants in horticulture by government agencies, universities, environmental organizations, and for-profit vendors. Additionally, some state and local governments have prohibited the sale and use of plants deemed to be invasive or noxious (e.g. Connecticut, Indiana, Massachusetts, Minnesota). Nonetheless, there has been some resistance from horticulturalists to remove invasive plants from their inventories. Peters et al. (2006) note that characteristics which make plants suitable for mass production in horticulture (rapid reproduction, hardiness) also increase their potential to become invasive. Additionally, consumer demand for familiar horticultural products and a lack of effective communication about what species are considered problematic can increase the likelihood that invasive species persist in horticultural catalogs.

Celastrus orbiculatus, commonly known as Oriental bittersweet, is a highly invasive ornamental woody vine (or liana) in the eastern USA. The species is widely recognized as a threat to native ecosystems because of its rapid growth that crowds out native vegetation, negatively affects forestry operation, and can alter natural successional trajectories (Fike and Niering 1999, Leicht-Young et al. 2007b). *Celastrus scandens* (American bittersweet, or American staff vine) is a congener native to the region that *C. orbiculatus* has invaded in North America. *Celastrus scandens* is also a woody vine, and is widely marketed as an ornamental alternative to *C. orbiculatus*. Judging from multiple personally communicated accounts from horticultural consumers, some concerns exist that plants marketed as *C. scandens* and "American bittersweet" are actually *C. orbiculatus*, or hybrids of the two species. Mislabeling may occur unintentionally, as it is difficult to distinguish the *Celastrus* species in the absence of reproductive structures (Leicht-Young et al. 2007a), and plants purchased from vendors are usually small individuals that have not begun to flower. Additionally, seeds collected from pistillate *C. scandens* may be sired by *C. orbiculatus* (Chapter 2), and hybrids of the two species have been found in the wild (Chapter 1). The problem of hybrid substitution for *C. scandens* will be greatest when the source of commercially available plants is seed collected from *C. scandens* in areas where *C. orbiculatus* co-occurs. Mislabeling may also occur intentionally, because *C. orbiculatus* grows more rapidly than *C. scandens* (thus increasing yields while decreasing investment of time and resources) and has a long history in horticulture, with multiple named varieties.

The problem of mislabeled products derived from plants and animals can be addressed with molecular wildlife forensics. Molecular wildlife forensics, the use of genetic markers to test for the presence or identity of non-human organisms, is a relatively new field that arose with the development of polymerase chain reaction (PCR) technology and the application of molecular markers to distinguish species, populations, or individuals (Ashley 1999). Molecular wildlife forensics has been applied widely, including legal cases involving patent violations (Congiu et

al. 2000) and murder investigations (Craft et al. 2007), studies of the illicit drug trade (Gilmore et al. 2003), and identification of rare or protected species used in animal-derived products (DeSalle and Birstein 1996). Several published studies test the accuracy of labels for products derived from plant material, including foods, medicines, and wood (reviewed in Zaya and Ashley 2012).

I used molecular forensics to test the species identity of commercially available plants marketed as *C. scandens*. My goal was to determine whether *C. orbiculatus* or hybrids were sold in place of *C. scandens*. If the ultimate source of marketed plants is seed collected from wild plants, it is possible that a large proportion of individuals are hybrids. Alternatively, *C. orbiculatus* may be substituted, intentionally or not, because the two species are difficult to distinguish morphologically in the absence of reproductive structures. Human commerce is amongst the most important dispersal agents of introduced species, and understanding its role is essential in any large scale attempt to control the spread of the introduced vine and its negative effect on natural communities, and *C. scandens* in particular.

3.2 Methods

3.2.1 Study species

Celastrus scandens L. (Celastraceae) is the only member of the genus native to North America (Hou 1955). It is usually found in open habitat. Its range extends from southern Quebec to South Dakota, south to western Texas through Georgia. The native range of *C. orbiculatus* Thunb. is in far eastern Asia, in Korea, Japan, and China (Hou 1955). It is found in thickets and lowland slopes, but can thrive in shaded habitat that would likely exclude *C. scandens* (Pavlovic and Leicht-Young 2011). Both species are usually dioecious, although rare individuals and populations displaying other breeding systems are known.

Celastrus orbiculatus was introduced as an ornamental vine to the eastern USA in the mid- to late-nineteenth century. The first reports of naturalization come from the early twentieth

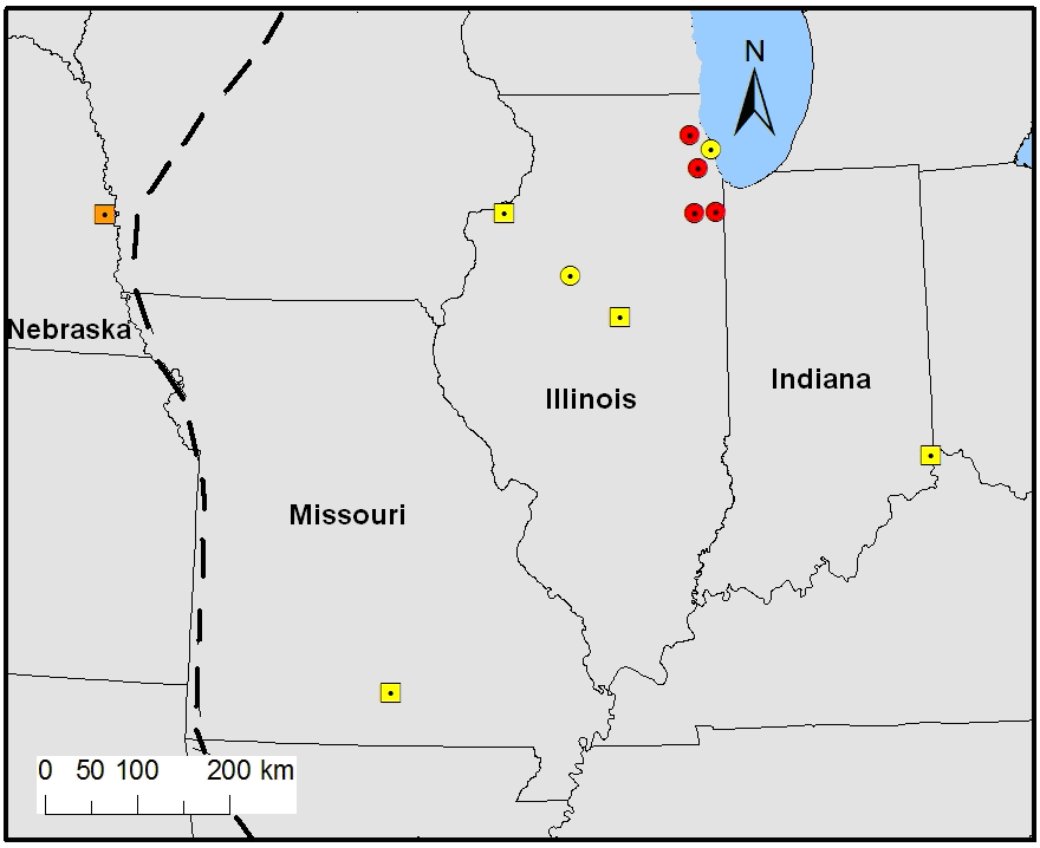
century; by the middle of the twentieth century it was widely recognized as a pest species rapidly spreading in the eastern USA (Patterson 1974). Land managers and foresters consider *C. orbiculatus* a troublesome species because it is a strong competitor that crowds out native vegetation, negatively affects forestry operation, and can alter natural successional trajectories (Fike and Niering 1999, Leicht-Young et al. 2007b). *Celastrus scandens* has been listed as a species of special concern in several states at the center of its range; in Delaware it may be extirpated, while it is listed as a vulnerable species in New York, a threatened species in Massachusetts, and an endangered species in North Carolina. There is strong evidence that *C. orbiculatus* interferes with successful reproduction in *C. scandens* through asymmetric pollen flow and hybridization (see Chapter 2), and it is likely that declines in *C. scandens* are linked to regions where invasion by *C. orbiculatus* is oldest and most extreme (Dreyer et al. 1987, Stewart et al. 2003, Leicht 2005, R. I. Bertin pers. comm.).

3.2.2 Sampling

I purchased plants marketed as "American bittersweet" or "*Celastrus scandens*" from 11 vendors in the Midwestern USA (in Indiana, Illinois, Missouri, and Nebraska; Fig. 3.1). Purchases were made in-person from six vendors, and the other five purchases were made over the Internet or by telephone. Sampling locations range across the invasion front of *C. orbiculatus* (EDDMapS 2012). The purchases were made in summer and fall of 2009. In total, 34 individuals were genetically tested, representing six named varieties, as well as plants not labeled with a variety name.

In addition to the nursery samples, I included three other types of control samples for genetic and statistical analysis. I used genetic benchmarks for *C. scandens* and *C. orbiculatus*, which were reproductive plants collected from the wild for which species identity could be determined using reproductive morphology, and whose species identity had been verified

Figure 3.1: Distribution of *Celastrus* vendors. Circles represent vendors which were visited in person, squares represent vendors that shipped the product. Red points represent vendors that exclusively sold *C. scandens*, yellow points represent vendors that exclusively sold *C. orbiculatus*, and the single orange point represents a vendor that delivered both species. The dashed line represents the approximate western edge of the *C. orbiculatus* invasion front (from EDDMapS 2012).



genetically. In total, I used 182 *C. scandens* individuals from 15 populations in nine states (Illinois, Indiana, Massachusetts, Michigan, Minnesota, North Carolina, Ohio, South Dakota, Wisconsin) and 180 *C. orbiculatus* individuals from 15 populations in nine states (Connecticut, Illinois, Indiana, Massachusetts, Michigan, North Carolina, New Jersey, Tennessee, Virginia) as benchmarks. Additionally, I included 16 hand-crossed hybrids from the Indiana Dunes National Lakeshore. These hybrids were hand-pollinated in the field, resulting seeds were collected, and put through cold stratification and germinated at the University of Illinois at Chicago greenhouse, according to the protocol outlined by Young and Young (1992).

3.2.3 Genetic analysis

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). For 32 samples, DNA was extracted from 20-25 mg of ground leaf material following the manufacturer's protocol. Two samples from the same supplier were not viable upon delivery, and did not have leaf material available. For those two samples I used 50-70 mg of scraped wood shavings for DNA extraction. I used a modified protocol developed by Rachmayanti et al. (2009), which included addition of polyvinylpyrolidone to the lysis buffer in order to help with DNA extraction from wood tissue. Five nuclear microsatellite loci were used to genotype each individual, where forward primers were synthesized with an M13 tail according the method describe by Schuelke (2000). These five loci can be used to distinguish the two species and their hybrids (Chapter 1). Nuclear microsatellites are especially useful for the objectives of this study because they are highly variable, making it possible to distinguish closely related species, and because they are codominantly inherited (one allele transmitted from each parent) which allows for accurate identification of hybrid individuals. PCR was conducted using 10-50 ng of genomic DNA in 10 µL volume containing 0.5 mM dNTP mix (Denville Scientific), 0.05 – 0.1 µM of the forward primer with the fluorescently labeled M13 (-21) universal primer extending from the 5' end, 0.33–0.6 µM of reverse primer, 0.16 µM fluorescently labeled M13 primer, 2.5 µg/ µL bovine serum albumin, and 0.025 U/ µL of Taq polymerase (Biotherm Taq; eEnzyme) with Biotherm

buffer (originally 10x, diluted to 1x). Thermal cycling conditions were as follows: 94ºC for 5 minutes, 35 cycles of denaturing (94ºC), annealing (see Table I for temperatures), and elongation (72 ºC) with each step lasting 30s, and a final extension at 72 ºC for 5 minutes. Fragment sizes of PCR product (using 1.0 – 1.5 µL) were analyzed with the ABI 3730 DNA Analyser, using a LIZ500 ladder (Applied Biosystems). All microsatellite genotypes were scored by analyzing the raw data using Applied Biosystem GeneMapper software, version 3.7.

3.2.4 Statistical analysis

Species assignments were evaluated using the program STRUCTURE version 2.3 (Falush et al. 2007). No *a priori i*nformation on species identity was included in the analysis. STRUCTURE implements a Bayesian clustering approach and Markov chain Monte Carlo simulations to provide estimates of an individual's identity with posterior probabilities. I used the admixture model assuming correlated allele frequencies and set the number of clusters, *K*, equal to two. I averaged data from three runs, each with 250,000 iterations after an initial burn-in of 50,000 iterations. Individuals were classified to one of the two species groups using the maximum posterior probability, *q*, of an individual genotype belonging to a single genetic cluster. If the maximum *q* was less than 0.85, an individual was categorized as a hybrid.

I used the nonparametric Mann-Whitney-Wilcoxon rank-sum test to compare prices of products identified as *C. orbiculatus* and *C. scandens*. The test was implemented in R version 2.15.1 (R Core Team 2012).

3.3 Results

Genetic tests indicated that 18 of 34 (53%) of the nursery samples clustered with *C. orbiculatus*. The other 16 samples all clustered with *C. scandens*. None of the samples clustered with hybrids. All of the STRUCTURE assignments were highly supported by the posterior probabilities. Every sample had a maximum posterior probability greater than 0.965, and all but one had a posterior probability greater than 0.99. Only two of the samples I

purchased showed signs of reproductive structures, both carrying fruits in terminal panicles typical of *C. scandens*. STRUCTURE correctly classified both samples as *C. scandens*.

Four of the 11 vendors sold only *C. scandens*. Six vendors sold only *C. orbiculatus*. The last vendor, located in Nebraska and beyond the *C. orbiculatus* invasion front, sold both species (Fig. 3.1). The mislabeled samples came under five varietal names: 'Diana', 'Hercules', 'Indian Brave', 'Indian Maiden', and 'Indian Mix'. The 'Diana' and 'Indian Maiden' varieties had identical genotypes, as did 'Hercules' and 'Indian Brave' (Table IV). The only named variety that was genetically determined to be *C. scandens* was 'Autumn Revolution' (aka *C. scandens* 'Bailum'), a relatively common product that was purchased from three of the five vendors that sold *C. scandens*. Six vendors sold plants that were not labeled with a varietal name, and in four of those cases genetic tests classified the samples as *C. orbiculatus*. One of the unnamed varieties had the same genotype as 'Diana' and 'Indian Maiden'. Table IV provides the multilocus genotypes of the named varieties as a reference to any reader interested in testing horticultural samples of unknown species identity. I found multiple samples with the same genotype due to asexual propagation. The identical genotypes included samples from the westernmost vendor in Nebraska and the easternmost vendor in southeastern Indiana. It is possible that others may find the same genotypes I report here.

Four of the six vendors that were visited in person exclusively sold *C. scandens*. All of the shipments included *C. orbiculatus*, a group that includes the vendor that sent both species (Fig. 3.1). The price of true *C. scandens* was more than twice the price of *C. orbiculatus* (Fig. 3.2), a significant difference ($W = 31$, $p < 0.04$). Interestingly, the vendor that sold both species charged more for *C. scandens* (19.95 USD) than mislabeled *C. orbiculatus* (13.95 USD).

3.4 Discussion

The majority of samples and named varieties that I tested, all marketed as "American bittersweet" or "*Celastrus scandens*," were mislabeled. Mislabeled *C. orbiculatus* is available on

both sides of the invasion front, and can easily be shipped to any state in the continuous USA. Some vendors also ship internationally, which could exacerbate the *C. orbiculatus* invasion in Canada and even in distant regions like New Zealand (Williams and Timmins 2003). None of the purchased samples were *C. scandens* x *C. orbiculatus* hybrids, as might be expected if wild-collected seeds were used for propagation. The lack of hybrids should not be a surprise, as both species are capable of prolific rhizomatous growth and the use of rootstock for propagation is a common practice in horticulture.

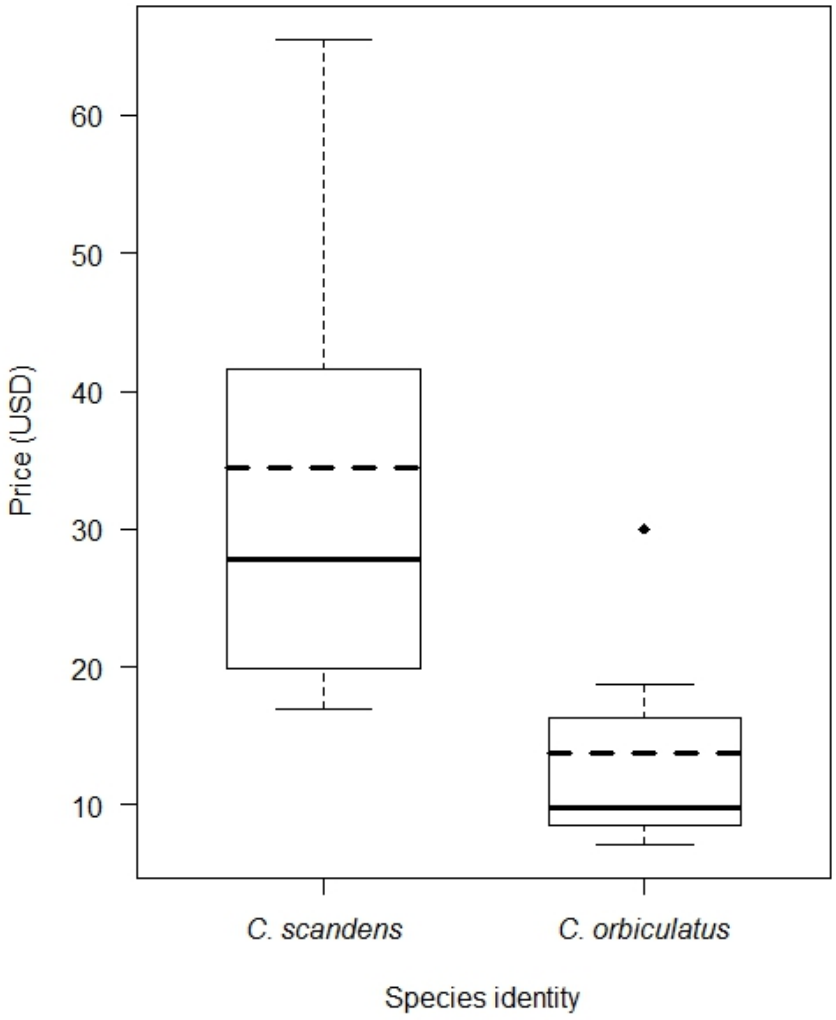
The large difference in price between mislabeled and correctly labeled products may be informative and important. The difference in price suggests that *C. orbiculatus* is easier to obtain or it can be propagated more efficiently. *Celastrus orbiculatus* has a long history in North American horticulture, and in many regions of the USA is more common than *C. scandens*. More efficient growth of mislabeled *C. orbiculatus* is consistent with ecological and physiological studies that have found *C. orbiculatus* to be a stronger competitor than *C. scandens*, exhibiting more rapid growth (Leicht-Young et al. 2011), tolerance of a larger range of conditions (Leicht-Young et al. 2007b), and greater reproductive output (Chapter 2, Dreyer et al. 1987). The lower price is important as it creates an incentive, even for well-meaning consumers, to purchase mislabeled *C. orbiculatus*. The case of the vendor that sold both species is revealing, as mislabeled plants cost 30% less. Thus, even when controlling for differences between vendors, mislabeled plants appear to be substantially less expensive. One positive aspect of the price difference is that it can be used as a hint of the true species identity of commercially available "American bittersweet". There was little overlap in the prices of the two species (Fig. 3.2).

Another useful clue as to the accuracy of product labeling might be the mode of purchase. Four of six in-person purchases were accurately labeled. Every vendor that delivered their product provided me with *C. orbiculatus*, though one of those vendors sold both species. All of the delivered products were initially found through Internet searches, though some were ordered over the telephone. Shifts in the purchasing patterns, towards increased

Table IV: Microsatellite genotypes for named *Celastrus* varieties. Individuals are diploid, with two alleles per locus. The numbers under each locus heading represent the length of the allele in base pairs, and alleles are separated by a slash. Note that the allele sizes used here depend on the usage of the method described by Schuelke (2000), and the same fluorescent label for each locus. The following dyes were used: NED – CESC006, VIC – CEOR7004 and CEOR7003, PET – CESC002 and CESC003.

				Microsatellite Loci				
Species	**Variety**	**Vendors**	**State**	**CESC002**	**CESC003**	**CESC006**	**CEOR7004**	**CEOR7003**
C.scandens	Autumn Revolution	3	IL	217/217	190/192	220/228	151/151	216/220
		1	IL	217/217	190/204	228/228	151/151	216/220
C. orbiculatus	Indian Mix	1	IL	235/239	214/222	201/219	159/173	243/243
				235/239	222/222	201/201	159/169	213/242
				235/235	220/222	201/201	169/173	242/248
	Diana / Indian Maiden	3	NE	231/235	220/222	219/226	169/169	227/232
	Hercules / Indian Brave	2	NE	219/231	220/220	201/219	169/173	242/242

Figure 3.2: Prices for *C. scandens* and *C. orbiculatus* purchased at Midwestern nurseries. For *C. scandens*, n = 5. For *C. orbiculatus*, n = 7. Dashed lines represent the mean, while heavy solid lines represent the median. A Mann-Whitney-Wilcoxon rank-sum test revealed significant differences (W = 31, p < 0.04).



online purchasing, may lead to an increase in the sales of incorrectly labeled *Celastrus*. Additionally, Internet and telephone purchases will increase the long-distance dispersal of *C. orbiculatus* through shipping. Vendors based on both sides of the invasion front can ship their products throughout North America. The mislabeled products can be shipped to areas where the sale and propagation of *C. orbiculatus* is illegal, such as the state of Minnesota or the city of Chicago, Illinois—where all of the products included in this study were shipped.

Properly labeled *C. scandens* appears to be difficult to obtain, even when purchasing from vendors that claim to sell "American bittersweet". All but one of the named varieties turned out to actually be *C. orbiculatus*. The one exception was Autumn Revolution, or *C. scandens* 'Bailumn', patented by Bailey Nurseries, Inc (St. Paul, MN). The availability of this product has increased recently, which means a properly labeled *C. scandens* can be easily obtained. However, overreliance on this variety may be troublesome. Autumn Revolution has the potential to become naturalized, as individuals readily set germinable seed which can be widely dispersed by birds. Also, plants grow more vigorously than typical *C. scandens*. Eight of the nine Autumn Revolution samples that I tested, from three different vendors, were genetically identical (Table. 3.1). The spread of the variety into the wild may threaten native *C. scandens* by decreasing genetic diversity of wild populations. Decreased genetic diversity may have several negative consequences, but in cultivated plants in particular it may lead to increased susceptibility to disease (Zhu et al. 2000). Also, the breeding system of Autumn Revolution is atypical in that all individuals have hermaphroditic flowers, while *C. scandens* is almost always dioecious. The potential for intraspecific crossing, long-distance pollen dispersal, and introgression between horticultural plantings and wild conspecifics has been demonstrated, and may threaten the genetic integrity of *C. scandens*. Johnson and Galloway (2008) provided evidence that individuals from natural *Lobelia cardinalis* populations were pollinated by horticultural *L. cardinalis* up to 1 km away, while Whelan et al. (2006) found the potential for introgression of unusual morphological characteristics from garden populations of *Grevillea*

macleayana into wild populations. If the use of Autumn Revolution is abandoned due to potential threats to wild *C. scandens* populations, it will be challenging to find an alternative. Only one vendor sold *C. scandens* plants that were not Autumn Revolution. That vendor, located in Monee, Illinois, sold an unnamed variety. Another named variety, 'Sweet Tangerine', is sparingly available commercially. However, this variety may also bear hermaphroditic flowers, and I have not tested its species identity.

The rate of mislabeling found in this study (63% of vendors, and 53% of samples tested) is large compared to previous studies that used molecular wildlife forensics to survey commercial plant products (Zaya and Ashley 2012). For example, several studies have tested the accuracy of labels on herbal medication and have reported mislabeling of 8-60% of the products tested (Mihalov et al. 2000, LeRoy et al. 2002, Del Serrone et al. 2006, Xue et al. 2006, Wang et al. 2007, Lin et al. 2008, Vongsak et al. 2008, Fan et al. 2009, Feng et al. 2010, Manissorn et al. 2010, Srirama et al. 2010). My study is unlike these examples and most others reported in the scientific literature, in that I tested viable plants capable of spreading into the wild, where they may affect wild populations and natural systems. Most reported studies test non-living material, which is usually meant for human consumption, with the exception of Honjo et al. (2008). The authors tested the reported source of stocks of an endangered plant species, *Primula sieboldii*. They found that at least 17% of the stocks they studied were not derived from the reported source populations, and argued that these stocks should not be used for restoration because they might alter the gene pool of locally adapted populations.

In the case of *Celastrus* in North America, what parties are responsible for the mislabeled samples, and can mislabeling be purely accidental? The two species are difficult to distinguish morphologically in the absence of flower or fruits (Leicht-Young et al. 2007a), and in most cases vendors are selling small plants that have not reached reproductive maturity. Thus, vendors that do not act as their own growers may not be to blame. The attention should turn to the growers that are the ultimate source of mislabeled plants. It is implausible that growers

propagating *C. orbiculatus* at a large scale and over an extended period of time may never observe the axillary inflorescences and yellow fruit capsules that readily distinguish the introduced vine from *C. scandens*, with its flowers in terminal panicles and orange fruit capsules. Hence, at some level, or levels, in the supply chain mislabeling is not accidental.

Horticulture created the invasion of *C. orbiculatus* in North America. The first introducers and propagators likely did not realize the potential for *C. orbiculatus* to spread in the wild, altering ecosystems and interfering with successful reproduction in a native congener. Nor did they likely espouse an understanding of biological invasions and the value of native planting that is increasingly the norm amongst scientists, horticulturalists, and citizens at large. Parties responsible for the propagation, and moreover the mislabeling, of *C. orbiculatus* today are aiding in the invasion of a known problematic weed and exacerbating the decline of a native species that could potentially be used as a horticultural substitute. One approach to curb the problem of mislabeled horticultural products is to attempt to institute penalties on dishonest suppliers through legal means. Another possible approach is to encourage self-policing. Both approaches have limitations, and lacking proper enforcement may lead to virtually no improvement in the situation. Dissemination of useful information in a manner accessible to the general public will help to create well-informed consumers, which will in turn help the problem, although it may not eliminate it.

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4. CARBON AND NITROGEN ENRICHMENTS CHANGE THE EFFECTS OF COMPETITION ON BIOMASS ALLOCATION IN A NATIVE (*CELASTRUS SCANDENS*) AND INVASIVE LIANA (*C. ORBICULATUS*)

4.1 Introduction

Anthropogenic alteration of the biosphere takes several forms which can have wide-ranging consequences for ecological systems. Two especially pervasive human-mediated changes are the alterations of the nitrogen and carbon biogeochemical cycles (Vitousek et al. 1997). Atmospheric carbon dioxide has increased by 40% over pre-industrial levels, and is expected to be double pre-industrial levels by the middle of the 21st century (Cox et al. 2000). Meanwhile, humans have more than doubled the production of biologically reactive nitrogen, a large proportion of which is lost to natural ecosystems through run-off or deposition (Galloway et al. 2008). The changes to these biogeochemical cycles can have a variety of large-scale consequences, including the relationship between elevated atmospheric carbon dioxide and climate change. Additionally, carbon dioxide and reactive nitrogen availability are both important for terrestrial, aquatic, and marine ecosystems and can have direct effects on ecological communities, such as altered net primary productivity (Schimel 1995, Elser et al. 2007). Different species and functional groups do not respond equally to carbon and nitrogen enrichment, thus changes in the cycles and availability of these elements can affect the balance of species in a community (Johnson et al. 1993, Suding et al. 2005).

Just as altered global biogeochemical cycles have the potential to affect plant communities throughout the world, increased human-mediated dispersal of species has increased the prevalence of invasive species. There has been a long history of interest in the role of nitrogen availability in successful invasions (Brooks 2000). Increasingly, attention has shifted toward the relationship between carbon dioxide enrichment and invasion (Dukes and Mooney 1999). The effects of increasing nitrogen and carbon dioxide on species and ecological communities do not necessarily act independently of one another (Tylianakis et al. 2008), but

the potentially complex interaction between carbon dioxide and nitrogen enrichment has rarely been addressed for invasive species (Vilà et al. 2007).

The two *Celastrus* species in North America provide an excellent opportunity to study the effect of nitrogen and carbon enrichment on invasiveness and competition. *Celastrus orbiculatus*, commonly known as oriental bittersweet, is a highly invasive liana (woody vine) in eastern North America. The species is widely recognized as a threat to native ecosystems because of its rapid growth that crowds out native vegetation, negatively affects forestry operation, and can alter natural successional trajectories (Fike and Niering 1999, Leicht-Young et al. 2007). *Celastrus scandens*, or American bittersweet, is also a liana, but it is native to the region that *C. orbiculatus* has invaded in North America. Comparisons of invasive taxa to closely related native species can be helpful in determining the causes and implications of successful invasion (Mack 1996, Daehler 2003). Previous experimental comparisons between the two *Celastrus* species have shown *C. orbiculatus* to be more tolerant of herbivory (Ashton and Lerdau 2008), tolerant of a wider range of moisture and light availability (Leicht-Young et al. 2007), capable of greater survival and growth rates, and less susceptible to negative density-dependent effects (Leicht-Young et al. 2011).

This study used a controlled greenhouse experiment to compare the response of *C. scandens* and *C. orbiculatus* to elevated levels of carbon dioxide and nitrogen, the interaction between the two factors, and how the factors affect the response to competition. I measured two groups of responses, those related to growth (total biomass and relative growth rate) and allocation (root-to-shoot ratio and a multivariate analysis of biomass of different tissues). The four primary objectives of this study were to determine whether: 1) the two species differ in their response to nitrogen fertilization, 2) the species differ in their response to elevated carbon dioxide, 3) carbon dioxide and nitrogen enrichment interact (have non-additive effects) on the response variables tested, and 4) elevated levels of carbon dioxide or nitrogen affect the competitive responses of *C. scandens* and *C. orbiculatus*. Studying the response of carbon

dioxide and nitrogen enrichment in the two species has the potential to elucidate the mechanisms behind the *C. orbiculatus* invasion, as well as to inform predictions of the future competitive dynamics of the two species.

4.2 Methods

4.2.1 Study species

Celastrus scandens L. (Celastraceae) is the only member of the genus native to North America (Hou 1955). It is usually found in open habitat. Its range extends from southern Quebec to South Dakota, south to western Texas through Georgia. *Celastrus scandens* has been listed as a species of special concern in several states in its range. In Delaware it is believed to be possibly extirpated, while it is listed as a vulnerable species in New York, a threatened species in Massachusetts, and an endangered species in North Carolina. The native range of *C. orbiculatus* Thunb. is in far eastern Asia, in China, Korea, and Japan (Hou 1955). It is found in thickets and lowland slopes, but can thrive in shaded habitat that would likely exclude *C. scandens* (Pavlovic and Leicht-Young 2011). *Celastrus orbiculatus* was introduced as an ornamental plant to the eastern USA in the mid- to late-nineteenth century. The first reports of naturalization come from the early twentieth century; by the middle of the twentieth century it was widely recognized as a pest species rapidly spreading in the eastern USA (Patterson 1974).

4.2.2 Experimental design

Thirty-six seedlings of each species were used in this experiment. I grew seedlings from seed collected at the Indiana Dunes National Lakeshore. Seeds were prepared for germination according to the protocol described by Young and Young (1992), which included cold stratification. Germination occurred in large flats in the University of Illinois at Chicago greenhouse. After germination, I randomly selected seedlings for inclusion in the experiment. I

also collected leaf tissue from each seedling in order to genetically verify its species identity (see Chapter 1 for details on the genetic and statistical methods).

Twelve seedlings for each species were potted singly in 25-cm diameter pots. The remaining 24 seedlings were grown in interspecific competition, with one seedling of each species in 25-cm diameter pots. The substrate used for potting was composed of equal parts sand and a growing mix of peat moss, perlite, and vermiculite (Growing Mix #2, Conrad Fafard Inc., Agawam, Massachusetts, USA). The growing mix included small amounts of fertilizer. The 24 competition pots were evenly and randomly divided to the *C. scandens* or *C. orbiculatus* portion of the study. Thus, in total there were 24 samples for each species, divided into 12 competition and 12 single-grown pots. Pots were watered daily, and rotated weekly to minimize spatial effects.

The samples were evenly divided into a fully-crossed design, with two carbon dioxide concentration treatments, two nitrogen treatments, and the two competition treatments described above. The carbon dioxide treatments were ambient concentration in 2011 (approximately 388 ppm) and elevated concentration (550 ppm). Samples in the low nitrogen treatment were fertilized with 500 mL of 5 mM ammonium nitrate solution, while the high nitrogen samples were fertilized with 500 mL of 50 mM ammonium nitrate solution. I fertilized samples in both treatments three days per week. In total, 48 replicates were included in the study, with three samples in each combination of species-competition-carbon dioxide-nitrogen treatment.

The experiment began on 27-28 July 2011. I measured the height for each focal individual at the beginning of the study on 1 August, and 74 days later on 14 October. I used the change in height to calculate the relative growth rate (RGR) for each individual, which is calculated as the log of the ratio in height (cm) divided by the length of the time interval in between measurements (months). The plants were harvested on 20-22 October 2011. For each focal individual the tissues were divided into four compartments (stem, leaf, fine root, and

coarse root), dried at 65°C for ten days, and weighed. The total biomass for each individual was calculated as the sum of the four categories. The root-to-shoot ratio was calculated as the quotient of the total belowground biomass (sum of fine and coarse roots mass) and the total aboveground biomass (sum of stem and leaf mass). Insecticidal sprays and soaps were applied to experimental subjects on three dates (22 August, 28 August, and 2 October) to control herbivore populations.

4.2.3 Data analysis

Due to heteroscedasticity associated with the large difference in growth rate between *C. scandens* and *C. orbiculatus* observed in this study and elsewhere (Leicht-Young et al. 2011), I analyzed the responses of the two species separately.

Relative growth rate (RGR) was analyzed with a generalized linear model (GLM) where the left-skewed response was modeled by a Gamma distribution. To eliminate negative values and allow modeling with a Gamma distribution, I transformed the RGR values by adding 0.03 to each value in *C. scandens* and 0.02 to each value in *C. orbiculatus*. The explanatory variables tested were competition treatment, nitrogen treatment, and carbon dioxide treatment, as well as all possible interactions (including the three-way interaction). Total biomass and log-transformed root-to-shoot ratio were both analyzed with linear models (factorial ANOVA), with the same three explanatory variables and interactions used for relative growth rate. Finally, the biomass of the four tissue compartments measured here (fine roots, coarse roots, stem, and leaf mass) was analyzed in a single analysis with a multivariate analysis of variance (MANOVA). Again, the same set of explanatory variables was used, and the species were separated in the analyses. Using Wilk's lambda or Pillai's trace did not affect the conclusions reached by the MANOVA, thus for simplicity I only present the results of Wilk's lambda.

All statistical tests were completed using R version 2.15.1 (R Core Team 2012). When using linear models (total biomass and log-transformed root-to-shoot ratio) or generalized linear models (RGR), I performed model selection by starting with a maximal model and proceeding

through backward elimination where the factor or interaction to be eliminated and tested next was determined with a random number generator (Crawley 2005). I did not attempt to remove factors from the model if they were included in a significant interaction. A cutoff of $\alpha = 0.05$ was used to determine whether or not to eliminate a factor.

4.3 Results

No explanatory variables significantly predicted RGR in either species, as the null model could not be rejected in either analysis. The results of model selection in *C. scandens* showed that the removal of every term yielded a $p > 0.15$. In *C. orbiculatus*, the removal of each term during model selection resulted in $p > 0.2$. In the analysis of total biomass the null model could not be rejected for either species, with the removal of each term resulting in $p > 0.2$. Thus, none of the factors I tested had a significant effect on biomass of *C. scandens* or *C. orbiculatus*.

There were significant treatment effects for the log-transformed root-to-shoot ratios. In *C. scandens*, there was a significant interaction between competition and carbon dioxide treatments (linear model, $R^2 = 0.267$, $p < 0.02$). At ambient levels of carbon dioxide *C. scandens* allocation to roots increases under competition, but at elevated levels of carbon dioxide competition lead to decreased relative root allocation (Fig. 4.1). In *C. orbiculatus*, the three-way interaction between competition, nitrogen, and carbon dioxide treatment was significant (linear model, $R^2 = 0.457$, $p < 0.02$). At low levels of nitrogen, the same effect of competition and carbon dioxide was observed as in *C. scandens*. Plants grown in competition at ambient carbon dioxide doubled their relative allocation to roots, while plants at elevated carbon dioxide decreased root allocation (Fig. 4.2). However, in the high nitrogen treatments, the effect of competition on *C. orbiculatus* was reversed in both carbon dioxide treatments (Fig. 4.2).

Figure 4.1: Relationship between root-to-shoot ratio and the interaction of carbon dioxide and competition treatments in *C. scandens*. Error bars represent one standard error (n = 6 for each category). The interaction between carbon dioxide and competition treatments significantly predicted the log-transformed root-to-shoot ratio (linear model, $R^2 = 0.267$, $p < 0.02$).

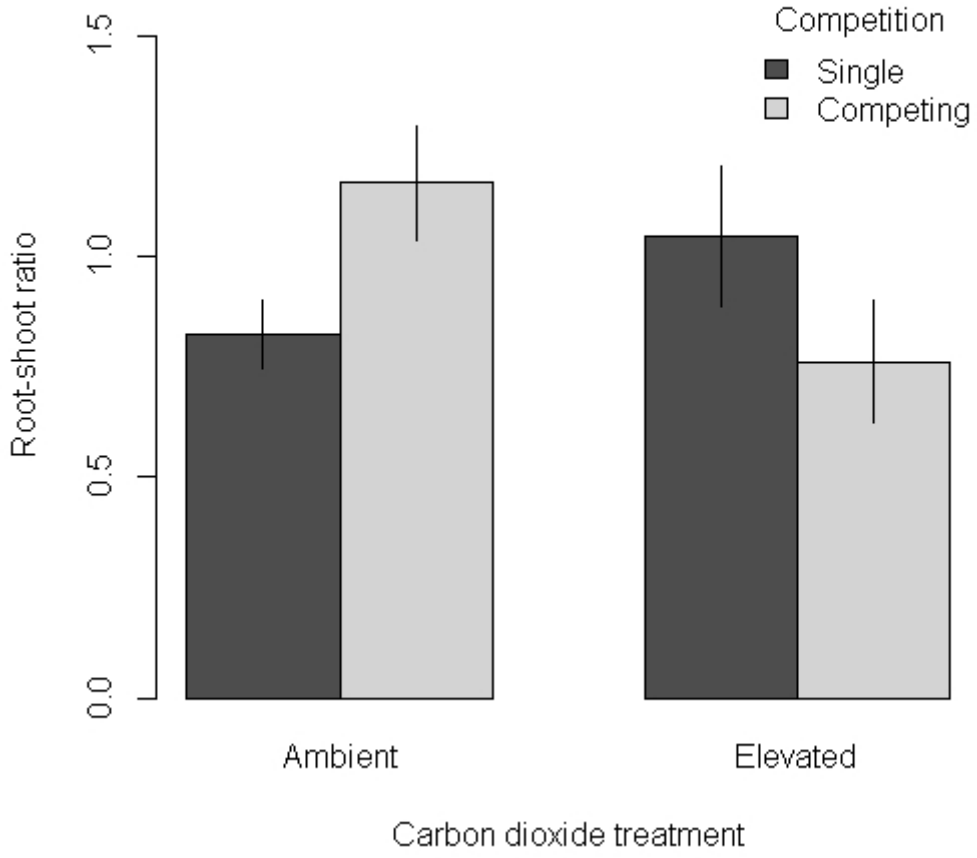
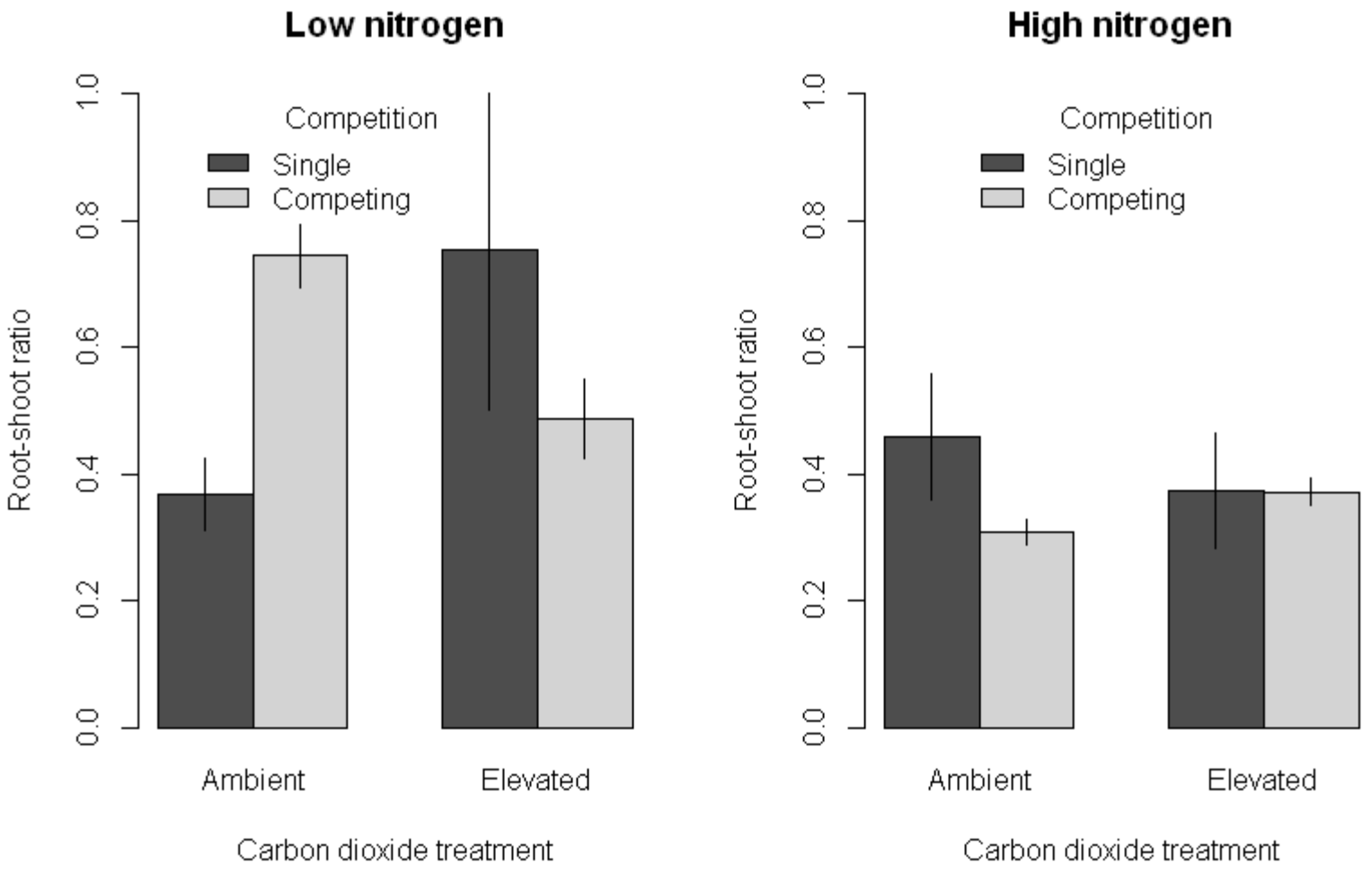


Figure 4.2: Relationship between root-to-shoot ratio and the three-way interaction of carbon dioxide, nitrogen, and competition treatments in *C. orbiculatus*. Error bars represent one standard error (n = 3 for each category). The three-way interaction of treatments significantly predicted the log-transformed root-to-shoot ratio (linear model, $R^2 = 0.457$, $p < 0.02$).



There was no significant effect of any of the factors or interactions on the multivariate response of allocation to compartment in *C. scandens*, though the interaction between competition and carbon dioxide approached significance (MANOVA, $p = 0.1$, Table V). For *C. orbiculatus*, the three-way interaction significantly affected biomass allocation to the different compartments (MANOVA, $p < 0.04$, Table VI), a result similar to the response in root-to-shoot ratio. Closer inspection of the univariate components showed that the strongest relationship was in coarse root mass with nitrogen ($p = 0.05$), and stem mass with the three-way interaction ($p = 0.086$).

4.4 Discussion

Neither measure of growth in *C. scandens* or *C. orbiculatus* significantly responded to carbon dioxide, nitrogen, or competition treatment. Nitrogen is a limiting resource for plant growth, and I expected a positive response in growth with nitrogen fertilization (Vitousek and Howarth 1991). The lack of response to nitrogen enhancement suggests that nitrogen was not a limiting resource for the subjects of this experiment, which could be due to the fact that nitrogen saturation was accomplished in both treatments or another factor was substantially more limiting than nitrogen. The lack of response to carbon dioxide, despite the fact that woody plants nearly always increase biomass under elevated carbon dioxide (Curtis and Wang 1998), also indicates that another factor was limiting plant growth in this experiment. Evidence for the role of factors not quantified in this experiment limits the conclusions that can be drawn regarding the relationship between growth and nitrogen, carbon dioxide, and the interaction of these two factors.

Both species showed significant changes in allocation to different tissues, and the significant factors differed between *C. scandens* and *C. orbiculatus*. For root-to-shoot ratio in *C. scandens* there was a significant interaction in carbon dioxide and competition (Fig. 4.1). At ambient levels of carbon dioxide allocation to roots increased, suggesting that below-

Table V: MANOVA summary for biomass of different tissues in *C. scandens*. The multivariate response variable included the mass of coarse roots, fine roots, stems, and leaves. The model tested three predictors (comp = competition treatment, c = carbon dioxide treatment, n = nitrogen treatment) and all possible interactions. Interactions are symbolized with an asterisk.

Factor	Wilk's lambda	Approx. F-statistic	df	P-value
comp	0.93927	0.21013	4, 13	0.9282
c	0.84549	0.59393	4, 13	0.6732
n	0.95983	0.13602	4, 13	0.9661
comp*c	0.57219	2.42998	4, 13	0.1004
comp*n	0.72806	1.21394	4, 13	0.3517
c*n	0.88183	0.43552	4, 13	0.7807
comp*c*n	0.75079	1.07877	4, 13	0.4066

Table VI: MANOVA summary for biomass of different tissues in *C. orbiculatus*. The multivariate response variable included the mass of coarse roots, fine roots, stems, and leaves. The model tested three predictors (comp = competition treatment, c = carbon dioxide treatment, n = nitrogen treatment) and all possible interactions. Interactions are symbolized with an asterisk.

Factor	Wilk's lambda	Approx. F-statistic	df	P-value
comp	0.71128	1.3192	4, 13	0.31416
c	0.80619	0.7813	4, 13	0.55706
n	0.57325	2.4194	4, 13	0.1014
comp*c	0.62825	1.9231	4, 13	0.1665
comp*n	0.81601	0.7328	4, 13	0.58566
c*n	0.77517	0.9426	4, 13	0.47017
comp*c*n	0.47751	3.5561	4, 13	**0.03601**

ground competition was especially important. Under elevated concentration of carbon dioxide competition increased allocation to shoots suggesting the prominence of above-ground competition, possibly related to light. In *C. orbiculatus*, the response of root-to-shoot ratio to carbon dioxide enrichment and competition treatment depended on nitrogen availability, where in the low-nitrogen treatment the same relationship was observed as in *C. scandens*. However, the relationships were reversed when plants were fertilized with additional nitrogen (Fig. 4.2). The other measure of biomass allocation, the multivariate response of biomass in four different tissue compartments (fine roots, coarse roots, stem, and leaf tissue), responded in a manner similar to root-to-shoot ratio, although the response was not quite statistically significant in *C. scandens* (Table V and Table VI).

While allocation in both species responded to different nutrient and competition treatments, the significant factors differed between *C. scandens* and *C. orbiculatus*. Specifically, there was no evidence of a significant response to nitrogen in *C. scandens*. This difference suggests that *C. orbiculatus* is more phenotypically plastic in response to nitrogen fertilization, which may be connected to its success in the invasion of the highly eutrophic terrestrial ecosystems of eastern North America. Nitrogen fertilization can significantly alter biomass allocation in plants because it can shift the dynamics of above-ground and below-ground resource limitation (Wilson 1988). Additionally, increased plasticity of invasive species has sometimes been offered as an explanation of why they can come to dominate in their introduced range (Richards et al. 2006). Although root-to-shoot ratio is generally expected to increase with fertilization, there was no response in *C. scandens*. Carbon dioxide can have complex and unpredictable effects on biomass allocation (Rogers et al. 1996), and the evidence from *C. orbiculatus* in this study suggests that the effect of carbon dioxide will depend largely on nitrogen availability.

Other studies of lianas under elevated carbon dioxide show that they may increase biomass production to a greater degree than most other plants (Schnitzer et al. 2008). The

pattern has been observed in a number of genera, *Hedera* (Zotz et al. 2006) and *Toxicodendron* (Mohan et al. 2006), although the net effects on biomass production have been disputed (Schnitzer et al. 2008). Additionally, a comparison of invasive and native *Lonicera* showed a likely advantage for the invasive species with future global change, although nitrogen enrichment was not included in the study (Sasek and Strain 1991).

The lack of growth response to nitrogen and carbon dioxide tempers any conclusions that can be drawn about the future of competitive response between these congeneric lianas and the effect of global climate change on invasiveness of *C. orbiculatus*. However, responses in allocation do provide evidence that carbon dioxide and nitrogen enrichment can interact in complex ways, and that those responses will not affect all species equally.

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APPENDIX

Table VII: Sample sizes, locations, and species identities of *Celastrus* across the USA. Asterisks next to a location name denote sites I personally sampled. Sample size corresponds to the putative species identity, while the number of unique genotypes applies to the species assignments determine genetically ("Genetic Assignment"). Abbreviations used: NHP (National Historic Park), NRA (National Recreational Area), NMP (National Memorial Parkway), NP (National Park), NBP (National Battlefield Park), NHS (National Historic Site), NM (National Monument), FP (Forest Preserve), SP (State Park).

Location	State	Putative Species	Sample Size	Genetic Assignment	Unique genotypes
Weir Farm NHS	CT	*C. orbiculatus*	10	*C. orbiculatus*	10
Bunker Hill *	IL	*C. scandens*	3	*C. scandens*	3
		Unknown	4	Hybrid	2
Bur Oak Woods *	IL	*C. orbiculatus*	2	*C. orbiculatus*	10
		Unknown	9	Hybrid	1
Dan McMahon Woods *	IL	Unknown	11	*C. orbiculatus*	11
Deer Grove *	IL	*C. orbiculatus*	3	*C. orbiculatus*	2
Greene Valley FP *	IL	*C. scandens*	6	*C. scandens*	4
		Unknown	3		
Harms Woods *	IL	*C. orbiculatus*	11	*C. orbiculatus*	10
Illinois & Michigan Canal State Trail *	IL	*C. scandens*	6	*C. scandens*	11
		Unknown	6		
Illinois Beach SP *	IL	*C. scandens*	21	*C. scandens*	10
Lyons Woods *	IL	*C. orbiculatus*	23	*C. orbiculatus*	17
Middlefork Savannah *	IL	Unknown	4	*C. orbiculatus*	2

Location	State	Putative Species	Sample Size	Genetic Assignment	Unique genotypes
Private property	IL	*C. scandens*	1	*C. scandens*	1
Ryerson Conservation Area *	IL	*C. scandens*	1	*C. scandens*	4
		Unknown	12	*C. orbiculatus*	2
				Hybrid	3
Waterfall Glen *	IL	*C. orbiculatus*	13	*C. orbiculatus*	11
				Hybrid	2
Wright Woods *	IL	Unknown	9	*C. orbiculatus*	2
Beemsterboer Natural Area	IN	*C. scandens*	1	*C. scandens*	2
		Unknown	1		
Clark and Pine Nature Preserve *	IN	*C. scandens*	12	*C. scandens*	10
Indiana Dunes National Lakeshore *	IN	*C. orbiculatus*	67	*C. orbiculatus*	54
		C. scandens	69	*C. scandens*	58
Abraham Lincoln Birthplace NHP	KY	*C. scandens*	1	*C. scandens*	1
Boston Harbor Islands NRA	MA	*C. orbiculatus*	10	*C. orbiculatus*	9
Worcester County,	MA	*C. orbiculatus*	1	*C. orbiculatus*	1
Utility right-of-way		*C. scandens*	2	*C. scandens*	2
		Intermediate	5	Hybrid	5
George Washington Memorial Parkway	MD	*C. orbiculatus*	3	*C. orbiculatus*	3
Sleeping Bear Dunes National Lakeshore	MI	*C. orbiculatus*	2	*C. orbiculatus*	2
(Mainland) *		*C. scandens*	23	*C. scandens*	25
		Unknown	3		

Location	State	Putative Species	Sample Size	Genetic Assignment	Unique genotypes
South Manitou Island (Sleeping Bear NL)	MI	*C. scandens*	9	*C. scandens*	9
		Unknown	1		
Warren Dunes SP *	MI	*C. orbiculatus*	25	*C. orbiculatus*	22
		C. scandens	26	*C. scandens*	17
				Hybrid	1
Pipestone NM	MN	*C. scandens*	15	*C. scandens*	4
Blue Ridge Parkway	NC	*C. orbiculatus*	5	*C. orbiculatus*	8
		C. scandens	3	*C. scandens*	2
		Unknown	9	Hybrid	6
Chimney Rock SP *	NC	*C. scandens*	33	*C. scandens*	2
Great Smoky Mountains NP	NC	*C. orbiculatus*	10	*C. orbiculatus*	5
		C. scandens	10	*C. scandens*	4
Homestead NM	NE	*C. scandens*	10	*C. scandens*	3
Morristown NHP	NJ	*C. orbiculatus*	9	*C. orbiculatus*	9
Hopewell Culture NHP	OH	*C. orbiculatus*	2	*C. orbiculatus*	2
		C. scandens	13	*C. scandens*	13
Gettysburg NMP	PA	*C. orbiculatus*	3	*C. orbiculatus*	3
		C. scandens	1	*C. scandens*	2
		Unknown	2		
Badlands NP	SD	*C. scandens*	10	*C. scandens*	10
Private property	SD	*C. scandens*	10	*C. scandens*	9

Location	State	Putative Species	Sample Size	Genetic Assignment	Unique genotypes
Wind Cave NP	SD	*C. scandens*	10	*C. scandens*	2
Cumberland Mountain SP *	TN	Unknown	2	*C. orbiculatus*	1
Sycamore Shoals SP *	TN	*C. orbiculatus*	11	*C. orbiculatus*	11
Warriors' Path SP *	TN	*C. scandens* Unknown	12 1	*C. scandens*	3
Guadalupe Mounatains NP	TX	*C. scandens*	10	*C. scandens*	2
Montgomery Hall Park *	VA	*C. orbiculatus* unknown	2 20	*C. orbiculatus* *C. scandens*	2 14
Richmond NBP	VA	*C. orbiculatus*	10	*C. orbiculatus*	10
Private property *	WI	*C. scandens*	12	*C. scandens*	9
		TOTALS	654		475

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